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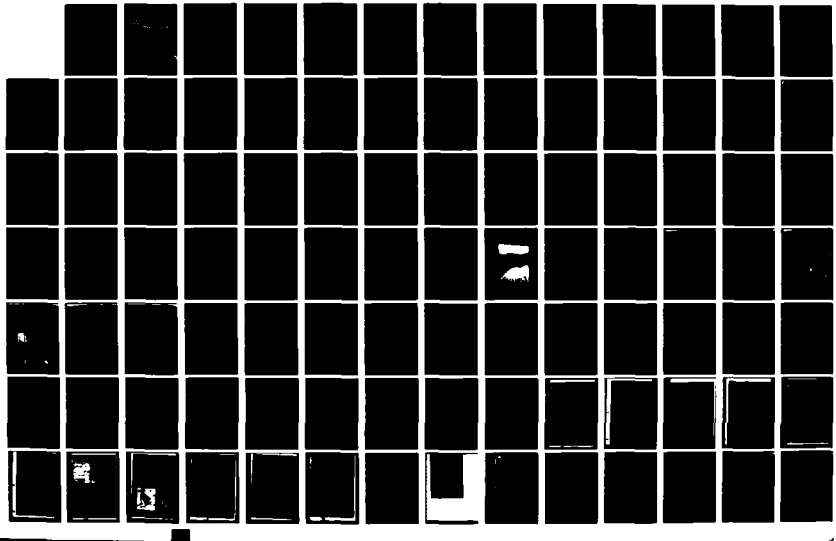

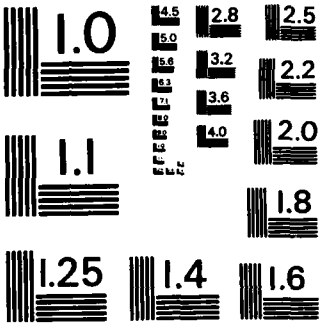

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CHEMICAL CARCINOGEN (HYDRAZINE et al.) INDUCED CARCINOGENESIS OF HUMAN DIPLOID CELLS, IN VITRO

George E. Milo
Department of Physiological Chemistry
and Comprehensive Cancer Center

For the Period
September 1, 1981 - August 31, 1982

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Polynuclear hydrocarbons or hydrazine analogues do not have an absolute requirement for binding to DNA in order to elicit a carcinogenic event. Furthermore, PNH like BP enter the nucleus via a lipoprotein complex where the BP is oxygenated and then interacts with the genetic material, (DNA). The adduct formed is 7β-BPDEI-dG. Benzamide does not alter the binding of the perceived ultimate carcinogen to dG but eliminates the carcinogenic response. The period of heightened response to the carcinogen appears to be 3 hours into

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Block 20 (Abstract) - Continued

S phase of the cell type. The modifying effect of TPA or Benzamide appears to alter the nuclear non-histone proteins protecting the cellular DNA.

Annual Technical Report

CHEMICAL CARCINOGEN (HYDRAZINE et al.) INDUCED CARCINOGENESIS OF HUMAN DIPLOID CELLS, IN VITRO

Inclusive Dates of Report: September 1, 1981 to August 31, 1982

The Ohio State University
Columbus, Ohio 43210

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Animal Use Statement

The experiments reported herein that will require care and maintenance here at OSU will be treated in accordance with principles and guidelines outlined in "Guide for the Care and Use of Laboratory Animals" prepared for the committee on Care and Use of Laboratory Animals, DHEW publication no. (NIH) 78-23 revised 1982.

George E. Milo

George E. Milo
Comprehensive Cancer Center

The Ohio State University has on file with the Office of Protection from Research Risks, NIH, a statement of assurance concerning the care and treatment of laboratory animals. This assurance states that the University complied with the NIH Guide for the Care and Use of Laboratory Animals, applicable portions of Public Law 91-579, and related rules and regulations issued by the Secretary of Agriculture.

Richard L. Wright
Deputy Director for Development
The Ohio State University
Research Foundation

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i) **Work Scope for the Fiscal Period Sept. 1, 1981 to Aug. 31, 1982**

a) To examine changes in histone labeling patterns or carcinogen-nuclear non-histone protein binding during the early and late stages of the carcinogenic process following exposure to different carcinogens.

b) To investigate the interaction of BP and/or BP metabolites with DNA during the induction process and correlate these adducts with BP oxygenated metabolites produced during the activation stage.

c) To investigate different nitrosamines for their carcinogenic activities.

d) To investigate the role of promoters (modulators) in the induction process.

e) To study the interaction of BP and/or BPDE-I with cellular DNA of responsive and refractory cell populations.

f) To investigate the role of the cell cycle in influencing chemical induction of human cell transformation.

g) To inevstigate the effects of hydrazines and their analogues on unscheduled DNA repair synthesis.

ii) **Progress on the Work Scope Sept. 1, 1981 to August 31, 1982**

Last year we were investigating the movement of benzo(a)-pyrene, BP, into the nuclei of responsive human fibroblasts. We found that the BP was transported to the nucleus via a lipoprotein complex. At the nuclear membrane, the BP-lipoprotein complex required energy to penetrate the nucleus. Once the radiolabel BP was in the nucleus, the complex between BP and the cytoplasmic carrier protein was altered. In the nucleus, BP forms a tighter complex with the nuclear protein. Preliminary results suggested that the BP had been oxidized to an oxygenated metabolite that formed a covalent bond with the nuclear protein. Separation of the nuclear proteins into histones and non-histone protein fractions revealed that the BP was tightly bond to the non-histone protein fraction. The kinetics of either methylation, acetylation or phosphorylation of the histones was not altered from the control untreated population during this 18 hr period of treatment. Furthermore, there were very little (non-detectable) levels of the radiolabel BP attached to the histone fraction, i.e. BP-histone bound.

When we used other carcinogens such as radiolabel 7R ⊖ BPDE-I, MNNG (a general alkylating carcinogen) or aflatoxin B_1, the radiolabel did not bind to the histone protein. Moreover, the use of [^{3}H]-TH-DMBA, (Cazer et al., 5, 499-506, 1981, Battelle Memorial Institute Symposium), 1,2,3,4,tetrahydro-7,12 dimethyl benz[a]anthracene induced a carcinogenic event in the treated cells. The significance of these results with TH-DMBA is connected with the behavior of TH-DMBA in the human cells. TH-DMBA has a reduced-A-ring that prevents the formation of a "bay region epoxide" and a "K-region epoxide". The formation of these epoxides has been suggested as an essential requirement for an ultimate polynuclear hydrocarbon (PNH) carcinogenic form. It is interesting to note that TH-DMBA is a better carcinogen than BP and is transported to the nucleus in the same manner that BP is transported, i.e. TH-DMBA-lipoprotein complex. We then chemically synthesized another analogue labeled CP-DMBA. In this case the compound was also transported to the nucleus in the same manner as either BP or

TH-DMBA. The significance of this compound and its activity is of interest because the A-ring is reduced to a 5 member ring thereby eliminating any possibility of artifact when these reduced PNH's are in the cell. The artifact would be associated with the intracellular removal of protons from the A-ring of the PNH and thereby create an unsaturated 7,12-DMBA molecule. Seven, 12-DMBA can be transported to the nucleus, however, it is not found localized in the nucleus, i.e. it is not translocated into the nucleus. TH-DMBA, BP and CP-DMBA are found localized in the nucleus. Each is bound tightly to a nuclear protein. Subsequent removal of the PNH's from this protein cannot be achieved by extraction with ethyl acetate. Moreover, when the nuclear PNH-protein complex was treated with acid (0.25 N HCL) the complex does not dissociate. When the complex is treated with sodium dodecyl sulfate at pH 5.5-7.0, the complex does dissociate. The radiolabel PNH is then recovered from the dissociated complex and when analyzed by HPLC it was found to be the parent non-oxygenated BP. The complex for transporting the BP in the nucleus following movement of the PNH from the lipoprotein cytoplasmic carrier into the nucleus is not lipoprotein in nature but a non-histone nuclear protein. We propose that this complex then interacts with the oxygenase system in the nucleus that oxygenates the BP to the ultimate carcinogenic form of BP.

The polynuclear aromatic hydrocarcbon (PNH) 1,2,3,4,-tetrahydro-7,12-dimethylbenz(a)anthracene, (TH-DMBA) was found to be a carcinogen when evaluated on human foreskin fibroblasts as measured by anchorage-independent growth and cellular neoplasia. Under conditions for transformation, radiolabeled [^{3}H-G]-TH-DMBA was examined for its biotransformation to oxygenated metabolites and intracellular distribution under conditions for transformation. Under these conditions we recovered 95% of the [^{3}H-G]-TH-DMBA as the non-oxygenated PNH. It also was interesting to note that [^{3}H-G]-TH-DMBA localized in the nucleus, 1.4 x 10^7 molcules/nuclear residue compared to 1.6 x 10^9 molecules per nuclear residue for [^{3}H-G]-benzo(a)pyrene (BaP) and non-detectable amounts per nuclear residue for [^{3}H-G]-7,12-dimethyl benzo(a)anthracene (DMBA). The transformation indices as measured by anchorage-independent growth were found to be for, BaP treated cells, 16-26; 7,12 DMBA 0; TH-DMBA 84-123; 2,3-dihydro-5, 11-dimethyl-1H-cyclopent a anthracene (CP-DMA), CP-DMA 18-21; BPDE-(I)anti 26-33. These data imply a mechanism for PAH-induced carcinogenesis in human cells that does not involve extranuclear activation as a prerequisite for biotransformation of the PAH to an ultimate carcinogenic form. If covalent binding is involved in PAH induced carcinogenesis, then less than 1% of the intracellular TH-DMBA associated with nuclear macromolecules is responsible for the alteration in nuclear function resulting in carcinogenesis. Therefore, we propose that there are a small number of critical sites in the human fibroblasts genome whose perterbation is essential to BaP genotypic modification which results in the expression of human cell carcinogenesis.

Turning our attention to, how BP is handled by cells in a confluent dense culture we treated both confluent dense and randomly proliferating populations with BP.

The metabolism of benzo(a)pyrene in randomly proliferating and confluent cultures of human skin fibroblast cells (non-transformable cells) was compared with cell cultures blocked in the G_1 phase of the cell cycle and released (transformable cells). When the cell populations were treated with (G-^{3}H) benzo(a)pyrene for 24 hrs. and the organic soluble metabolites in the extracellular medium were analysed by HPLC, no significant metabolism was observed in the transformable cell populations. There was an eight fold increase in metabolism in the confluent, non-

transformable cell populations which do not transport benzo(a)pyrene into the nucleus. The trans-7,8-dihydroxy-7,8-dihydro-benzo(a)pyrene, trans-9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene and 3-hydroxy benzo(a)pyrene were the major metabolites formed. Small amounts of the sulphate conjugate, the 9-hydroxy-benzo(a)pyrene, the 7,10/8,9-tetrol-I, the 7/8,9,10-tetrol-I and the 7,9,10/8-tetrol II were also detected.

These results indicate that the induction of neoplastic transformation in the normal human skin fibroblast cells by benzo(a)pyrene may not require metabolic activation of the parent polynuclear hydrocarbon at the plasma membrane level, (Tejwani et al., Molecular Pharmacology, Submitted, 1982, (copy enclosed).

iii) **Results From Collaborative Efforts:**

Examination of the association of the oxygenated BP and/or 7 β -BPDE-I with cellular DNA was evaluated. The uptake of benzo(a)pyrene (BP) by low passage (LP) and high passage (HP) human skin fibroblast cells is followed by its transport into the nucleus as the parent compound. When the LP and HP cells were treated with BP for 24 h and the DNA was isolated and enzymatically digested, several DNA adducts were detected. In both the LP and HP cells a small amount of the radiolabel was associated with the 7β BPDE-I-dG, 7α -BPDE-I-dG and BPDE-II-dG adducts. Although there were no major qualitative differences in the adducts formed in the LP and HP cells, a higher proportion of the radiolabel was associated with the 7β -BPDE-I-dG adduct in the LP cells. When LP or HP cells were treated with BPDE-I, the ultimate carcinogenic form of BP, similar levels of DNA modification were observed in the two cell types and the h.p.l.c. profiles of these adducts were essentially identical. BPDE-I induced a carcinogenic event in the LP but not the HP cells as measured by anchorage independent growth in soft agar and ceulular invasiveness of the chick embryonic skin organ cultures. (Tejwani et al., Carcinogenesis, 3:727-732, 1982).

An intriguing result(s) was observed following 7β -BPDE-I treatment of the fibroblast. There was further metabolism of the BPDE-I into BPDE-II and 7α -BPDE-I. All of the analogues reacted with dG.

We found, serendipitously, that 1-NO_2-pyrene can be reduced enzymatically to 1-NH_3-pyrene and it interacts with dG in the DNA. It is not converted to biamino or bisamio or other amino-pyrene derivatives. This compound transforms human diploid fibroblasts in vitro. (Howard et al., Carcinogenesis, submitted 1982, Howard et al., Ffth CIIT Conference on Toxicology: Toxicity of Nitroaromatic Compounds, In Press, 1982).

There has been considerable interest in recent years concerning the metabolism, mutagenicity and carcinogenicity of nitroarenes, especially nitrated polycyclic aromatic hydrocarbons (PAHs). Part of this concern is a result of data from a number of laboratories which demonstrate that nitrated PAHs may be environmental contaminants. Moreover, reports have stated that nitrated PAHs, and in particular nitrated pyrenes, are present in carbon black toners. These observations, coupled with the demonstration that some of these compounds are potent mutagens, point to the need for detailed studies on the metabolic activation of nitrated PAHs. We have found that nitrated PAHs may be reduced by mammalian nitroeductases, to N-hydroxyamino-PAHs and that these N-hydroxy arylamines could subsequently bind to DNA. Based on this premise, we have studied the in vitro activation of 1-nitropyrene by the purified mammalian

nitroreductase, xanthine oxidase. In particular, we have established that this enzyme will catalyze the reducation of 1-nitropyrene to a DNA-binding species. The major DNA adduct has been characterized and shown to be identical to the major bound product in Salmonella typhimurium which were incubated with 1-nitropyrene. Finally, we determined that xanthine oxidase will increase the transformation frequency of diploid human fibroblasts when they are incubated with either 1-nitrophyrene or 6-nitrobenzo(a)pyrene under anaerobic conditions.

Another collaborative effort established this year with Dr. Kun, University of California San Francisco, has led to our initial understanding how to further manipulate the carcinogenic events with a compound benzamide, (BZ). Methylanzoxyethanol, (MAMA), was used to tritrate the carcinogenic response by human fibroblasts and the paper will be presented at the Conference on "Human Cell Carcinogenesis" at Aspen Colorado on Sept. 16-21, 1982. BZ has been used to interfere with MAMA elicited carcinogenic responses, the results of which are presented here:

Human foreskin fibroblast populations blocked in G_1, released and treated with methyl azoxy methanol acetate (MAMA) from the time of release (late G_1) for 1 hr treatment intervals until 4 hr into S, exhibited a differential sensitivity to MAMA treatment at the different treatment times. A heightened response to the carcinogen treatment was not detected until calmodulin, a cell regulatory protein, was optimally present in the nuclei of the late G_1 treated cells 6 hrs after release from the G_1 block. Moreover, there was a distinct increase in the number of transformed phenotypes, (cells that will grow in soft agar) observed when the cells were treated with MAMA at the onset of scheduled DNA synthesis. The time at which these treated cells were optimally responsive to a carcinogenic insult was 12-13 hrs after release from the block 2-3 hr into S. Interestingly, this was followed by a decrease in the expression of anchorage indpendent growth when the cells were treated 13-14 hrs after release from the block 4 hrs into S. Benzamide interfered in the process when added at the onset of S and the resultant carcinogen treated population did not exhibit a comparable increase in expression of anchorage independent growth. Cells treated with MAMA at the point of release from the block G_1^{0-1} to G_1^{3-4} did not express anchorage independent growth.

It is my contention that the heightened presence of calmodulin in the nuclei 4 hrs prior to the onset of scheduled DNA synthesis is a cell regulatory function that sets in motion a complex series of events, (program) in carcinogen initiated human fibroblasts that leads to a subsequent carcinogenic response.

Dr. Kun's Laboratory in San Francisco has found a striking and sustained increase in poly ADP-ribosylation of non-histone proteins in the chemical carcinogen induced precancerous site and found recently that the promoter process of carcinogenesis itself chronologically coincides with this increase of poly ADP-ribosylation even in the absence of a carcinogen. It was postulated that selective inhibition of poly-ADP-ribosylation should prevent carcinogenesis by removing the DNA repair inhibiting effect of increased poly ADP-ribosylation. This prediction was verified with a variety of carcinogens using human fibroblast transformation as a bioassay.

These undoubtedly biologically significant results demonstrate a readily recognizable function of poly(ADP-R) in cell biology that has an immediate significanace in cancer chemotherapy, since it is predictable that carcinogenic action of chemotherapeutic agents will be abolished by the simultaneous administration of benzamide.

TABLE III (cf. 37)

Prevention of carcinogensis in human fibroblasts by lmM benzamide, a specific inhibitor of poly(ADP-R) polymerase.

No.	Treatment of cells **	No. of cancer cell colonies formed/50,000 cells
1.	Methylazoxy-methanol-acetate	300 - 350
2.	1 + benzamide	1
3.	N-me-N-nitrosoguanidine	45 - 50
4.	3 + benzamide	1
5.	3-hydroxy-l-propane sulfonic acid -propiolactone	40 - 42
6.	5 + benzamide	1
7.	Benzamide	1

** Carcinogenesis was induced by exposing human fibroblast after release from G_1 block for 10-14 hours to 1 μM carcinogens. The preventive effect of benzamide runs paralell with its inhibitory effect of poly(ADP-R) synthetase, which enzyme system is greatly increased at this stage.

The biological function of poly(ADP-R) based on its macromolecular properties is envisaged as a nucleic acid component of a cross linking system, capable of promoting or inhibiting the regulatory effect of chromatin proteins on transcription. Two examples, the action of triiodothyronine and of chemical carcinogens illustrate this complex action of the homopolymer functioning as a protein modifier.

Although DNA and RNA can be profitably studied in isolated systems without paying attention to poly(ADP-R), integration with cellular physiology makes it mandatory to include poly(ADP-R) as a nucleic acid that possesses exclusively regulatory function, (Kun, et al., Steinbock-Lily Symposium, Madison WI, In Press, 1982). These events associated with an increase in phosphorylation of non-histone nuclear proteins appears to be the common factor where the correct modification of the preceding events results in a carcinogenic response. Furthermore, ^{14}C-MAMA-DNA adduct formation in BZ treated cells with BZ non-treated ^{14}C-MAMA treated cells was strikingly similar. However, cells treated with MAMA only exhibited 700 colonies in soft agar per 10^5 seeded cells whereas when BZ was added we observed < 2 colonies per 10^5 seeded cells. Our work with Dr. Kadlubar continues on the co-carcinogen experiments. Data will be ready for our future reports. These results at the molecular level i.e. carcinogen-DNA adducts formation in the presence and absence of BZ suggest that DNA adduct formation is not the critical event.

Dr. Witiak's laboratory is making excellent progress on the DNA-hydrazine, MAMA effort.

Cell cycle studies to study the effects of phorbol esters on cell cycle activation using the published cytofluorometric procedures developed for animal cell systems were not translatable to human cells. Dr. D. Tomei had to abandon the published techniques designed for animal studies and reevaluate the application of published systems to human cell kinetic cell cycle work. The vital stains used to stain cellular DNA would not work. We are continuing to evaluate these stains as described above. Either scheduled DNA synthesis in human cells undergoes a multiple initiation point of the beginning of DNA synthesis or the intercalating DNA dyes do not penetrate the nucleus in the same manner as that described for animal cells. By modifying our isolation procedures we now are obtaining yields in excess of 50% clean nuclei. The application of these techniques will permit us to investigate the toxic effects of compounds on the nuclei of the layers of skin grown in vitro.

We have found that nitrosamines, breakdown products of methylated hydrazine derivatives or biotransformation products of the hydrazine derivatives, will transform the cells.

iv) **Investigation in Progress**

1) We are continuing to investigate how the ultimate carcinogenic forms interact with cellular DNA. We are presently examining the DNA-carcinogen adducts that are formed under conditions that react in a toxic response versus a carcinogenic response.

2) We are asking why, once an adduct is formed with DNA, does the cell population in G_1 repair the lesion as an error repair response, while DNA damage repaired while the cells are in S results in a carcinogenic response. Moreover, cells that are pretreated with BZ at the onset of scheduled DNA synthesis followed by carcinogen treatment 4 hrs into S repair see the lesion as an error free repair response.

3) We are continuing our effort to investigate why some cells are refractory to the carcinogen treatment and why some cells are responsive to the treatment. Why is the program of cell cycle and its relationship to a carcinogenic response important?

v) Papers Published in 1981-1982 Sponsored by AFOSR

1. Donahoe, John, I. Noyes, G. Milo, and S. Weisbrode. (1982) A comparison of expression of neoplastic potential of carcinogen-transformed human fibroblasts in nude mice and chick embryonic skin. In Vitro. 18:429-434.

2. Howard, Paul, F. Beland, F. Evans, R. Heflich, G. Milo and F. Kadlubar. (1982) 1-Nitropyrene: In-Vitro metabolism and DNA formation, Fifth CIIT Conference on Toxicology: Toxicity of Nitroaromatic Compounds. In Press.

3. Tejwani, Raman, A. Jeffrey and G. Milo. (1982) Benzo(a)pyrene diol epoxide DNA adduct formation in transformable and nontransformable human foreskin fibroblast cells in vitro. Carcinogenesis. 3:727-732.

4. Howard, Paul, J. Gerard, G. Milo, P. Fu, F. Beland and F. Kadlubar. (1982) Neoplastic transformation of normal human skin fibroblasts by 1-nitropyrene and 6-nitrobenzo(a)-pyrene. Carcinogenesis, In Press.

5. Tejwani, Raman, R. Trewyn and G. Milo. (1981) Kinetics of movement of benzo(a)pyrene into transformable and nontransformable diploid fibroblasts. Chemical Analysis and Biological Fate: Polynuclear Aromatic Hydrocarbons, Fifth International Symposium, pp. 97-107.

6. Cazer, Frederick, M. Inbasekaran, J. Loper, R. Tejwani, D. Witiak and G. Milo. (1981) Human cell neoplastic transformation with benzo(a)pyrene and a bay region reduced analogue of 7,12-dimethylbenz(a)anthracene. Chemical Analysis and Biological Fate: Polynuclear Aromatic Hydrocarbons, Fifth International Symposium, pp. 499-507.

7. Milo, George, J. Oldham, R. Zimmerman, G. Hatch and S. Weisbrode. (1981) Characterization of human cells transformed by chemical and physical carcinogens in vitro. In Vitro. 17:719-729.

8. Milo, George and R. Trewyn. (1982) In vitro transformation of cultured human diploid fibroblasts. Banbury Report. pp. 23-33.

vi) **Papers to be Published in 1982-1983 Sponsored by AFOSR**

1. Milo, G., P. Fuhrer and A. Hamburger. (1983) Production of monoclonal antibody against human skin keratinocytes that can be used as phenotypic marker. Experimental Cell Research.

2. Kun, F. and G. Milo. (1983) Action of benzamide as a specific inhibitor of molecular events associated with methylanzory-methanolacetate induced cellular transformation. Proceedings of the National Academy of Science (USA).

vii) **Presentations, Abstracts 1982-1983**

4/27-5/3/82	Presented paper entitled "Epithelial Human Cell Transformation In Vitro" to the American Association of Cancer Research Association's annual meeting.
6/3/82	Presented a lecture at the OVBICA Annual Spring dinner meeting in Springdale, OH entitled "Human Cell Transformation in vivo - in vitro."

March 3, 1982

1-NITROPYRENE: *IN VITRO* METABOLISM AND DNA ADDUCT FORMATION

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Abbreviated title: 1-Nitropyrene adduct formation

Abbreviations used: PAHs, polycyclic aromatic hydrocarbons; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; Bis-Tris, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; MEM, Eagle's minimal essential media; HBSS, Hank's balanced salt solution; FBS, fetal bovine serum

There has been considerable interest in recent years concerning the metabolism, mutagenicity and carcinogenicity of nitroarenes, especially nitrated polycyclic aromatic hydrocarbons (PAHs). Part of this concern is a result of data from a number of laboratories which demonstrate that nitrated PAHs may be environmental contaminants (1-6). Moreover, Lofroth et al. (7) and Rozenkranz and coworkers (8) have reported that nitrated PAHs, and in particular nitrated pyrenes, are present in carbon black toners. These observations, coupled with the demonstration that some of these compounds are potent mutagens (4,8-12), point to the need for detailed studies on the metabolic activation of nitrated PAHs.

Four major findings concerning nitroarene metabolism have recently been reported. First, in the Salmonella reversion assay nitrated PAHs appear to be less mutagenic in nitroreductase-deficient Salmonella typhimurium strains than in normal tester strains (4,8,10-12). This suggests that reduction of the nitro moiety is required for the mutagenic activation of these compounds. Second, a number of nitroreductases have been identified in mammalian cells. These include cytosolic xanthine oxidase (13-16), DT diaphorase (17), and aldehyde oxidase (18), as well as microsomal cytochrome P-450 reductase (13,14,19) and cytochrome P-450 (13). Third, nitroreductases have been shown to reduce nitroaromatics to their corresponding N-hydroxy arylamines (18,20-24). Fourth, many aromatic amines and nitrofurans are activated to macromolecular-binding species through N-hydroxy arylamine metabolites (25-28).

These data suggest that nitrated PAHs may be reduced by mammalian nitroreductases, to N-hydroxyamino-PAHs (Figure 1) and that these N-hydroxy arylamines could subsequently bind to DNA. Based on this premise, we have studied the in vitro activation of 1-nitropyrene by the purified mammalian

nitroreductase, xanthine oxidase. In particular, we have established that this enzyme will catalyze the reduction of 1-nitropyrene to a DNA-binding species. The major DNA adduct has been characterized and shown to be identical to the major bound product in Salmonella typhimurium which were incubated with 1-nitropyrene. Finally, we determined that xanthine oxidase will increase the transformation frequency of diploid human fibroblasts when they are incubated with either 1-nitropyrene or 6-nitrobenzo[a]pyrene under anaerobic conditions.

MATERIALS AND METHODS

Chemicals

[4,5,9,10-^{3}H]1-Nitropyrene (specific activity, 117 mCi/mmole; radiochemical purity >99%) was obtained from Dr. Robert W. Roth, Midwest Research Institute, Kansas City, MO. Hypoxanthine, allopurinol, xanthine oxidase (Grade I), bovine-liver catalase, superoxide dismutase (Type I), deoxyribonuclease-I (Type DN-Cl), alkaline phosphatase (Type III-S), acid phosphatase (Type I) and bis(2-hydroxyethyl)amino-tris(hydroxymethyl)-methane (Bis-Tris) were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclease-P_1 (Grade A) was obtained from Calbiochem-Behring Corp. (LaJolla, CA). All other chemicals were reagent grade or better.

Incubation Conditions and DNA Adduct Isolation

The conditions for the incubations has been described previously (29). Briefly, 0.05, 0.1, or 0.5 U/ml xanthine oxidase was added to an argon-purged solution of 50 mM potassium phosphate, pH 5.8, containing 3.7 mM hypoxanthine, 2 mg/ml calf thymus DNA and 20 µM [^{3}H]1-nitropyrene. Following incubation at 37° for 1 hr, the reaction was terminated by addition of an equal volume of chloroform:isoamyl alcohol:phenol (24:1:25). The DNA was then purified as previously described (29) and its specific radioactivity determined.

A large scale (500 ml) incubation consisting of 0.10 U/ml xanthine oxidase, 20 µmoles [^{3}H]1-nitropyrene, 2 mg/ml DNA, and 10 µg/ml catalase was conducted to provide a quantity of DNA adducts sufficient for structural characterization. The solution was incubated for 2.5 hrs, and then additional aliquots of xanthine oxidase and hypoxanthine were added. After 18 additional hrs of incubation, the DNA was purified (29), precipitated with ethanol, dissolved in 5 mM Bis-Tris-HCl, pH 7.1, and enzymati-

cally hydrolyzed as described previously (30). The hydrolysate was applied to a Sephadex LH-20 column (2.5 x 15 cm). The salts, protein, and unmodified nucleosides were eluted with water and the DNA adduct fraction was eluted with methanol. The adduct fraction was dried in vacuo, reconstituted in methanol, and subjected to HPLC analysis using a reversed-phase μBondapak-C_{18} column with the water-methanol gradient shown in Figure 4. Fractions (1 ml) were collected and the radioactivity in each determined by scintillation counting. Typically, >95% of radioactivity applied to the column was recovered.

Incubation of Salmonella Typhimurium with [^{3}H]1-Nitropyrene and Characterization of the DNA Adduct(s)

A culture of Salmonella typhimurium TA98 (obtained from B.N. Ames, University of California, Berkeley, CA) was grown overnight in brain-heart infusion broth medium, then diluted 5-fold in the same medium. The bacterial suspension was purged with argon and then [^{3}H]1-nitropyrene (specific activity, 0.97 mCi/mmol) was added to a final concentration of 40 μM. The solution was incubated at 37° for 4 hrs. The bacteria were then harvested by centrifugation at 10,000xg for 10 min, and the DNA was isolated as previously described (31). The DNA was enzymatically hydrolyzed (30) and the adducts extracted with n-butanol. The adduct fraction was dried in vacuo, reconstituted in methanol, and subjected to HPLC as described above.

Incubation of Cultured Diploid Human Fibroblasts with 1-Nitropyrene

Cultures of normal human foreskin fibroblasts were prepared and treated as described previously (32-34). The cells, <6 population doublings, were propagated in Eagle's minimal essential medium (MEM) containing Hank's balanced salt solution (HBSS), 25 mM N-2-hydroxyethyl-

piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.2, supplemented with 10% fetal bovine serum (FBS). For transformation assays, the fibroblasts were inoculated into glutamine-, and arginine-deficient Dulbecco's modified MEM supplemented with 10% dialyzed FBS. When DNA replication ceased, the medium was changed to MEM supplemented with arginine, glutamine and 20% FBS. Then, after 12 hrs, the medium was changed to argon-purged MEM-HBSS, pH 7.2, containing 40.5 μM 1-nitropyrene or 33.7 μM 6-nitrobenzo[a]pyrene in acetone. To this were added 3.7 mM hypoxanthine and 0.1 U/ml xanthine oxidase, with or without 36.7 μM allopurinol in argon-purged MEM-HBSS, pH 7.2. Following a 2.5 hrs incubaton, the medium was removed and the cells maintained for 9.5 hrs in MEM-HBSS supplemented with 20% FBS. The cultures were then serially passaged 1:2 into 8x selection medium (32). After 20 population doublings, 5×10^4 cells were seeded into 25 cm^2 dishes containing 0.33% agar in supplemented Dulbecco's LoCal medium (Biolabs, Northbrook, IL), layered over a base of 2% agar in RPM1 medium 1629 (GIBCO, Grand Island, NY). Colony formation was evaluated after 21 days.

Instrumentation

^{1}H-Nuclear magnetic resonance (NMR) spectra were recorded in methanol-d_4 or dimethyl sulfoxide-d_6 (Aldrich, Milwaukee, WI) with a Bruker WM-500 spectrometer. UV/visible spectra were recorded with a Gilford Model 2400-2 spectrophotometer. Radioactivity was determined in Scintisol (Isolabs, Akron, OH), using a Searle Mark III scintillation spectrometer. High pressure liquid chromatography (HPLC) was performed using a Waters Associates System consisting of two Model 6000A pumps, 660 solvent programmer, U6K injector, and a 440 UV detector.

RESULTS

In Vitro Incubation of 1-Nitropyrene with Xanthine Oxidase and DNA

Incubation of [^{3}H]1-nitropyrene with xanthine oxidase resulted in high levels of covalent binding of this mutagen to added DNA. The binding increased in a linear manner for up to 80 min (Figure 2), was dependent upon the presence of xanthine oxidase and xanthine (or hypoxanthine), and could be inhibited in a concentration-dependent manner by the xanthine oxidase inhibitor, allopurinol (Table 1). In agreement with published data (35), the oxidation of xanthine or hypoxanthine to uric acid had a pH optimum >7 (Figure 3). In contrast, the xanthine oxidase-coupled binding of [^{3}H]1-nitropyrene to DNA was greatest at pH 5.5-6.0. All subsequent incubations were performed at pH 5.8 with 0.10 U/ml xanthine oxidase.

Nitroreductases are able to reduce not only nitroarenes and nitrofurans, but can also reduce molecular oxygen to superoxide (37), which will dismutate to hydrogen peroxide. Both superoxide and hydrogen peroxide have been shown to inactivate xanthine oxidase (37). In accord with these observations, 1.0 and 10 mM hydrogen peroxide inhibited [^{3}H]1-nitropyrene binding to DNA by 87 and 99%, respectively (Table 1). However, inclusion of superoxide dismutase in the incubation had no effect on the binding, whereas catalase increased adduct formation by 224%. Catalase in the absence of xanthine oxidase did not catalyze binding.

Structural Identification of the 1-Nitropyrene-DNA Adduct

In order to determine the structure of the 1-nitropyrene-DNA adduct(s), a large scale incubation was conducted. After multiple solvent extractions and DNA precipitations, the DNA was enzymatically hydrolyzed to mononucleosides. The adduct fraction was isolated by LH-20 chromatography and HPLC analysis of this mixture (Figure 4a) indicated one major adduct

(10 min) and two minor products (6 and 7 min). The major peak was isolated in sufficient quantity for structural determination.

High-field ^1H NMR analysis of the major adduct indicated the presence of all nine aromatic protons associated with the pyrene moiety (Figure 5). The resonances were assigned by comparison to the spectrum of 1-aminopyrene and through extensive homonuclear decoupling studies. These data signified substitution through the pyrene nitrogen. Examination of the spectrum also suggested the base to which the pyrene was attached. Each of the non-exchangeable deoxyribose protons could be located, therefore substitution through the deoxynucleoside base was indicated. The absence of signals which could be attributed to pyrimidines (2.4 and 5.9 ppm for thymidine, 6.0 and 8.0 ppm for cytosine; Ref.38), or deoxyadenosine (8.2 and 8.4 ppm) suggested a deoxyguanosine adduct. Since only resonances associated with pyrene were detected between 7.9 and 8.5 ppm the spectrum was consistent with substitution through the C8 position of deoxyguanosine. To provide further evidence for this assignment, NMR measurements were also conducted in dimethyl sulfoxide-d_6 which allowed observation of the exchangeable protons. Five additional resonances were detected. The single proton signals at 11.0 and 8.96 ppm, and the two proton resonance at 6.41 ppm, were quite similiar to those reported for N-(deoxyguanosin-8-yl)-2-amino-fluorene (10.6 ppm, deoxyguanosine-N1; 8.77 ppm, aminofluorene-N^2; 6.47 ppm, deoxyguanosine-N^2; Ref.39). The remaining two resonances were the sugar hydroxyls at 5.26 and 5.38 ppm. Thus, the NMR spectra were consistent with the major adduct from the xanthine oxidase-catalyzed incubation being N-(deoxyguanosin-8-yl)-1-aminopyrene.

Confirmation for this structure was obtained through application of the technique of Moore and Koreeda (40). Both acidic and basic pKa's were

detected (Figure 6); the presence of a basic pKa indicated that the product could not be substituted through the N1 or O^6 positions of deoxyguanosine (41). Furthermore, the pH-partitioning curve was quite similar in shape to those observed with other C8-arylamine-substituted deoxyguanosines (26,39). Aliquots of the adduct were then treated with either 0.1 N NaOH or 0.1 N HCl for 30 min at 37°. Under basic conditions (Figure 4b), a new product was formed that coeluted with one of the minor adducts at 6 min. When treated with acid, the adduct decomposed to give three compounds (Figure 4c), the first two of which correspond to the minor adducts from the xanthine oxidase incubation. The instability of the adduct under both acidic and basic condition is consistent with C8-deoxyguanosine substitution (26,30).

Incubation of 1-Nitropyrene with Salmonella Typhimurium

Salmonella typhimurium strain TA98 was incubated with 40 μM [^{3}H]1-nitropyrene for 4 hrs. The bacterial DNA was then isolated by multiple solvent extractions and hydroxyapatite chromatography. Following enzymatic DNA hydrolysis, the adducts were partitioned into n-butanol and analyzed by HPLC. As indicated in Figure 4d, the major adduct coeluted with the major bound product from the xanthine oxidase incubations. A second band of radioactivity migrated slightly after the major adduct and did not coelute with any of the in vitro products.

Incubation of Diploid Human Fibroblasts with 1-Nitropyrene and Xanthine Oxidase

When 1-nitropyrene was incubated with normal human diploid fibroblasts under anaerobic conditions, an increased transformation frequency was detected (Table 2). Incubations conducted in the presence of oxygen resulted in no transformants. Addition of xanthine oxidase and hypo-

xanthine to the anaerobic medium dramatically increased the transformation frequency and this enhancement could be abolished by coincubation with the xanthine oxidase inhibitor, allopurinol.

The same trends were noted when incubations were performed with 6-nitrobenzo(a)pyrene. Colony growth in soft agar was not observed when the fibroblasts were exposed to 6-nitrobenzo(a)pyrene under aerobic conditions, whereas colonies were detected in anaerobic incubations. As before, inclusion of xanthine oxidase and hypoxanthine in the mixture significantly increased the transformation frequency, and this increase was inhibited by allopurinol.

DISCUSSION

Nitroreduction has been implicated as an essential step in the mutagenic activation of nitropyrenes. This reduction would result in the successive formation of nitroso-, hydroxylamino- and amino-pyrenes. N-hydroxy arylamines are reputed to be the activated mutagenic metabolites of nitrofurans (18,20,42), several hair-dye components (43), and 4-nitroquinoline-1-oxide (17,44). In this study, we have demonstrated that xanthine oxidase can be used as a model nitroreductase to reduce 1-nitropyrene to metabolites that bind to DNA. In preliminary experiments, we have also established that the xanthine oxidase/xanthine system will also catalyze the reduction of 6-nitrobenzo[a]pyrene, 2-nitrofluorene, and 2,4- and 2,6-dinitrotoluene (Howard et al., in preparation).

The pH-dependence of the DNA binding by the xanthine oxidase-catalyzed 1-nitropyrene reduction product suggests that the reactive metabolite is N-hydroxy-1-aminopyrene. N-Hydroxy arylamines undergo acid-catalyzed decomposition to electrophilic resonance-stabilized nitrenium ions (41,45).

Since electrophile formation is acid-catalyzed, one would predict that the reactivity of N-hydroxy arylamines with DNA would increase 10-fold for each unit the pH is lowered. In practice, however, binding of several of the N-hydroxy derivatives has been reported to increase 5-fold/pH unit (25,30). When the experimental reactivity of N-hydroxy arylamines is added to the pH-dependent activity of xanthine oxidase (35), a theoretical curve can be formed for the xanthine-oxidase mediated reduction of nitroarenes to the DNA-binding metabolite. As shown in Figure 3, this theoretical curve agrees quite closely in shape and pH optimum to that observed experimentally for the reduction and binding of 1-nitropyrene to DNA.

Xanthine oxidase has been used to study the reduction of several compounds including nitroimidazoles and nitrobenzenes. Reduction of nitroimidazoles or nitrobenzenes occurs by a one electron pathway as evidenced by an electron spin resonance signal for the nitroanion, $R\text{-}NO_2^{\cdot}$ (46-48). Mason et al. (47) have suggested that the nitroanion either reduces molecular oxygen to superoxide or disproportionates to the nitroso derivative. Oxygen, therefore, would decrease nitroso formation with a concomitant inhibition of hydroxylamine production. In addition, the spontaneous dismutation of superoxide at acidic pH would result in the formation of hydrogen peroxide which has been shown, along with superoxide, to inhibit xanthine oxidase (37). Thus, the presence of oxygen in this model nitroreductase system could inhibit the reduction and subsequent DNA binding of 1-nitropyrene by three mechanisms: (1) direct reduction of oxygen by xanthine oxidase, (2) oxidation of the nitroanion with molecular oxygen being converted to superoxide, and/or (3) inhibition of the enzyme by superoxide or hydrogen peroxide. When catalase was included in the xanthine oxidase-1-nitropyrene incubation, DNA binding increased 2- to

3-fold (Table 1). This is indicative of hydrogen peroxide formation during the reduction sequence and is supportive of a 1-nitropyrene radical anion being formed. Superoxide dismutase had no effect upon the binding but this may be because under slightly acidic conditions superoxide will spontaneously dismutate to hydrogen peroxide.

Examination of the DNA revealed three adducts and the major product was characterized as N-(deoxyguanosin-8-yl)-1-aminopyrene. This product is also characteristic of N-hydroxy arylamine formation because C8-deoxyguanosine substitution has been observed with the N-hydroxy arylamines of 2-aminofluorene (39), 2-naphthylamine (26), N-acetylbenzidine (30), and 4-aminobiphenyl (49). The two minor adducts were more polar than the major product and may be ring-opened imidazole derivatives as has been noted for a number of C8-deoxyguanosine-substituted arylamines (26,30,49,50).

The potential significance of xanthine oxidase-mediated DNA damage by 1-nitropyrene was investigated by examinating the adducts formed in the Salmonella typhimurium reversion assay. When Salmonella typhimurium strain TA98 was incubated with 1-nitropyrene, an adduct was detected that had chromatographic properties identical to N-(deoxyguanosin-8-yl)-1-aminopyrene, the major adduct from the xanthine oxidase incubations. This observation is in agreement with the recently reported data of Messier et al. (51), who found that S. typhimurium reduced 1-nitropyrene to 1-aminopyrene with concomitant DNA binding. Our results also lend support to the concept that nitroreduction is essential in the mutagenic activation of 1-nitropyrene.

The carcinogenicity of 1-nitropyrene has recently been reported by Ohgaki et al. (52) who found malignant fibrous histiocytomas at sites of injection in male F344/DuCrj rats. We have found that under anaerobic

conditions, both 1-nitropyrene and 6-nitrobenzo(a)pyrene could cause the *in vitro* transformation of normal human fibroblasts to an anchorage-independent stage (i.e., growth in soft agar). Since transformants were not observed when the incubations were conducted aerobically, these data imply that the fibroblasts are capable of reducing both compounds to active metabolites. When xanthine oxidase and hypoxanthine were included in the incubation medium, a dramatic increase in colony forming frequency in soft agar was noted, and this increase was abolished by coincubation with the xanthine oxidase inhibitor, allopurinol. The increase in transformation frequency with xanthine oxidase provide support for the importance of nitroreduction as a critical step in the metabolic activation of nitrated PAH's. Interestingly, inclusion of allopurinol in the incubation mixture did not completely inhibit colony formation which suggests that human fibroblasts might contain additional nitroreductases capable of activating these compounds. A similiar mechanism of activation may be occurring when tumors are formed at sites of application (52).

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TABLE 1

EFFECT OF ALLOPURINOL, HYDROGEN PEROXIDE, CATALASE AND SUPEROXIDE DISMUTASE ON XANTHINE OXIDASE CATALYZED 1-NITROPYRENE-DNA BINDING

Incubation Conditions[a]	[^{3}H]1-Nitropyrene-DNA Binding % of Control ± S.D.[b]
Allopurinol, 0.73 µM	100.8 ± 24.5
" , 2.2 µM	66.4 ± 6.1[c]
" , 4.4 µM	51.5 ± 4.0[c]
Hydrogen Peroxide, 0.01 µM	94.9 ± 9.1
" , 1 µM	12.7 ± 3.1[c]
" , 10 µM	0.9 ± 0.0[c]
Catalase, 10 µg/ml (- xanthine oxidase)	0.1 ± 0.0[c]
" , 10 µg/ml	224.4 ± 14.6[c]
Superoxide Dismutase, 10 µg/ml	115.6 ± 28.3
Superoxide Dismutase, 10 µg/ml + Catalase, 10 µg/ml	199.7 ± 29.6[d]

[a]Incubation mixture consisted of 50 mM potassium phosphate, pH 5.8, containing 20 µM [4,5,9,10-^{3}H]1-nitropyrene, 3.7 mM hypoxanthine, 2 mg/ml DNA and 0.10 U/ml xanthine oxidase.

[b]Incubations were conducted 1 hr at 37°. The extent of binding was determined as described in Materials and Methods and represents the mean ± standard deviation of at least 3 separate incubations.

[c]Significantly different from control at $p < 0.05$ by Student's t-test.

[d]Significantly different from control at $p < 0.10$ by Student's t-test.

TABLE 2

EFFECT OF XANTHINE OXIDASE ON COLONY FORMATION IN SOFT AGAR INDUCED BY 1-NITROPYRENE OR 6-NITROBENZO[A]PYRENE

Conditions[a]	Frequency of Colony Growth in Soft Agar ± S.D.[b]
1-Nitropyrene	14 ± 6
+ Xanthine Oxidase, Hypoxanthine	81 ± 10
+ Xanthine Oxidase, Hypoxanthine, Allpurinol	10 ± 4
Control[c]	0
6-Nitrobenzo[a]pyrene	36 ± 9
+ Xanthine Oxidase, Hypoxanthine	62 ± 9
+ Xanthine Oxidase, Hypoxanthine, Allopurinol	10 ± 4
Control[c]	1 ± 1

[a]Normal human fibroblasts in Dulbecco's MEM with HBSS, pH 7.2, were treated with either 1-nitropyrene (40.5 μM) or 6-nitrobenzo[a]pyrene (33.7 μM) in the presence of xanthine oxidase (0.10 U/ml) and hypoxanthine (3.7 mM) with or without allopurinol (36.7 μM) for 2.5 hr. All incubations were performed under an argon atmosphere.

[b]Treated cell populations were serially passaged for 20 population doublings and then seeded into 2 ml of 0.33% agar containing growth medium over 5 ml 2.0% agar base. Colonies, 50 cells or greater in size were scored 21 days following seeding and the frequency determined per 10^5 cells. Each value is the mean ± standard deviation from six 25 cm^2 wells receiving 5×10^4 cells.

[c]Control conditions were MEM-HBSS only.

FIGURE LEGENDS

Figure 1 - Proposed pathway for the reduction of 1-nitropyrene by xanthine oxidase.

Figure 2 - Xanthine oxidase catalyzed binding of 1-nitropyrene to DNA. The incubation contained 50 mM potassium phosphate, pH 5.8, 3.7 mM hypoxanthine, 2 mg/ml calf thymus DNA, 0.5 U/ml xanthine oxidase and 20 μM [^{3}H]1-nitropyrene. The incubations were terminated at the times indicated and the extent of binding determined as described in Materials and Methods. Each point represents the mean ± standard deviation of at least three separate incubations.

Figure 3 - Effect of pH on xanthine oxidase activity and the binding of 1-nitropyrene to DNA. The incubation contained 50 mM potassium phosphate, 3.7 mM hypoxanthine, 2 mg/ml calf thymus DNA, 0.1 U/ml xanthine oxidase and 20 μM [^{3}H]1-nitropyrene. The incubations were terminated after 1 hr and the extent of binding determined as described in Materials and Methods. The xanthine oxidase oxidation of hypoxanthine to uric acid was determined spectrophotometrically (36). The theoretical 1-nitropyrene binding curve was constructed as described in the text.

Figure 4 - Reversed-phase HPLC profiles of a) xanthine oxidase-catalyzed 1-nitropyrene DNA adducts, b) major 1-nitropyrene DNA adduct treated with 0.1 N NaOH for 30 minutes at 37°, c) major 1-nitropyrene DNA adduct treated with 0.1 N HCl for 30 minutes at 37°, and d) DNA adducts formed in Salmonella typhimurium TA98 incubated with [^{3}H]1-nitropyrene. The methanol-water gradient used to separate the products is shown in panel a). Synthetic N-(deoxyguanosin-8-yl)-1-aminopyrene was added to the S. typhimurium DNA hydrolysate to serve as a UV marker.

Figure 5 - 500 MHz ^{1}H NMR spectrum and resonance assignments of N-(deoxyguanosin-8-yl)-1-aminopyrene in methanol-d_4 (~300 µg/ml) recorded at 32°. Data acquisition conditions were as follows: 416 80° pulses at 6.55 second intervals; sweep width, 5,000 Hz; data size, 64 K. Chemical shifts are reported in ppm downfield from tetramethylsilane.

Figure 6 - Partitioning of 1-nitropyrene-DNA adduct as a function of pH. The method of Moore and Koreeda (40) was used to evaluate the major adduct obtained from the xanthine oxidase incubations. The organic phase was 5% n-butanol in diethyl ether.

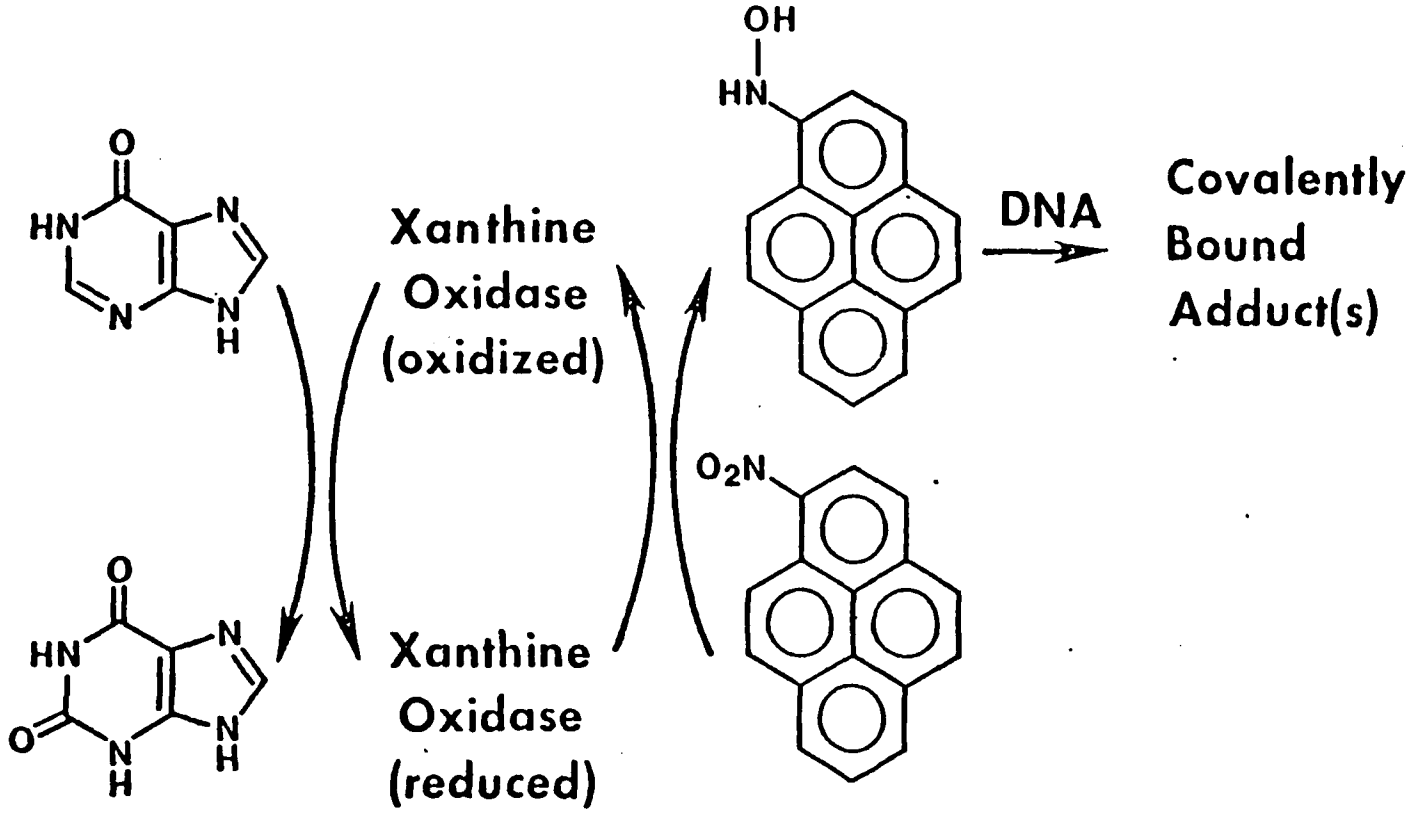


Figure 1 - Proposed pathway for the reduction of 1-nitropyrene by xanthine oxidase.

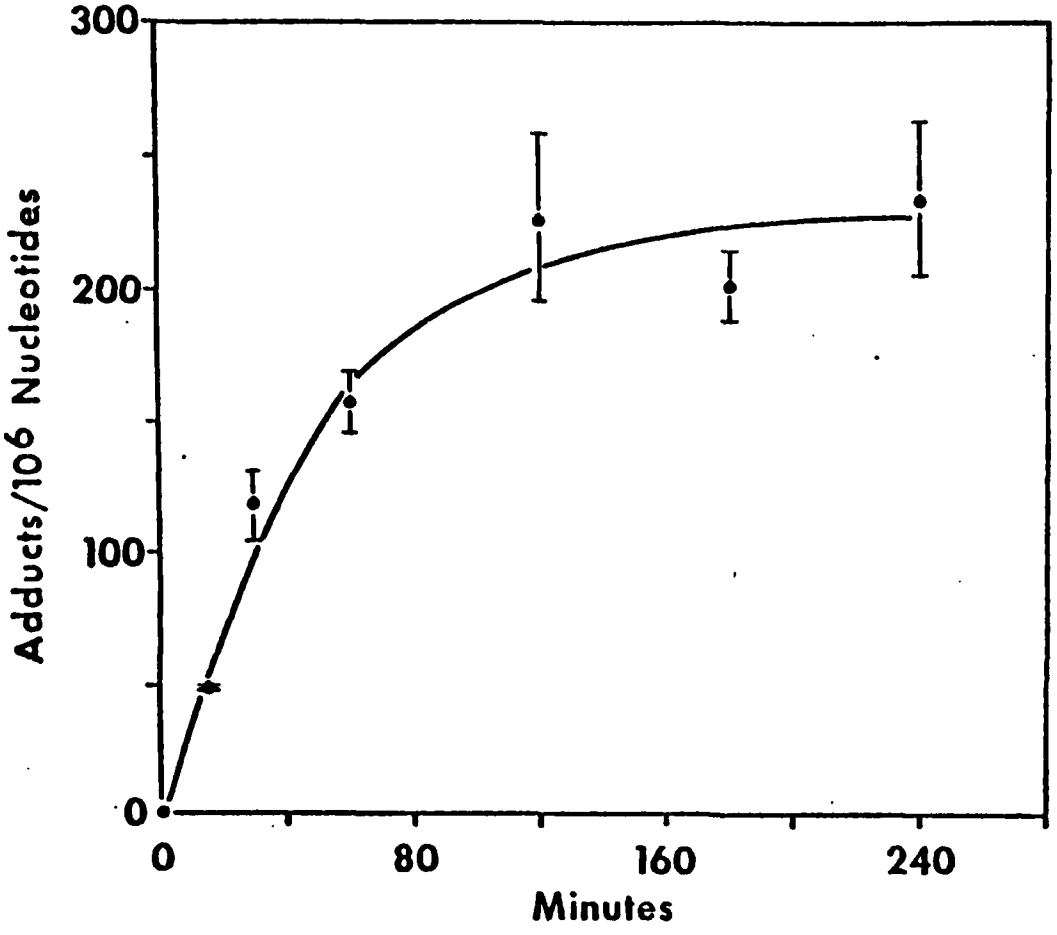


Figure 2 - Xanthine oxidase catalyzed binding of 1-nitropyrene to DNA. The incubation contained 50 mM potassium phosphate, pH 5.8, 3.7 mM hypoxanthine, 2 mg/ml calf thymus DNA, 0.5 U/ml xanthine oxidase and 20 μM [^{3}H]1-nitropyrene. The incubations were terminated at the times indicated and the extent of binding determined as described in Materials and Methods. Each point represents the mean ± standard deviation of at least three separate incubations.

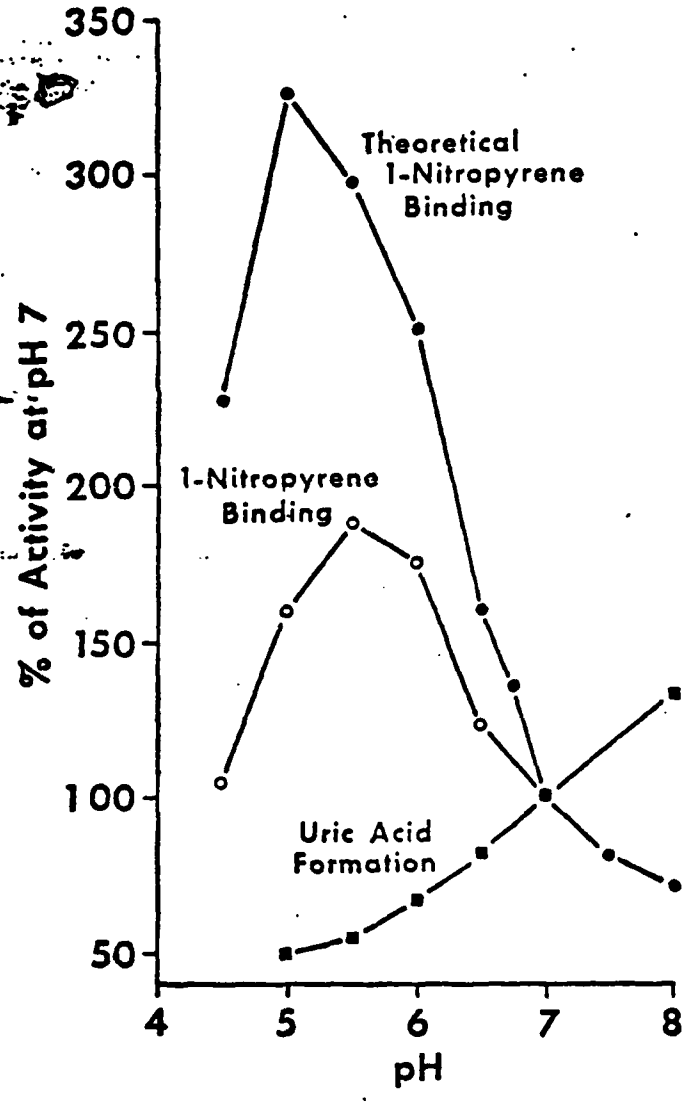
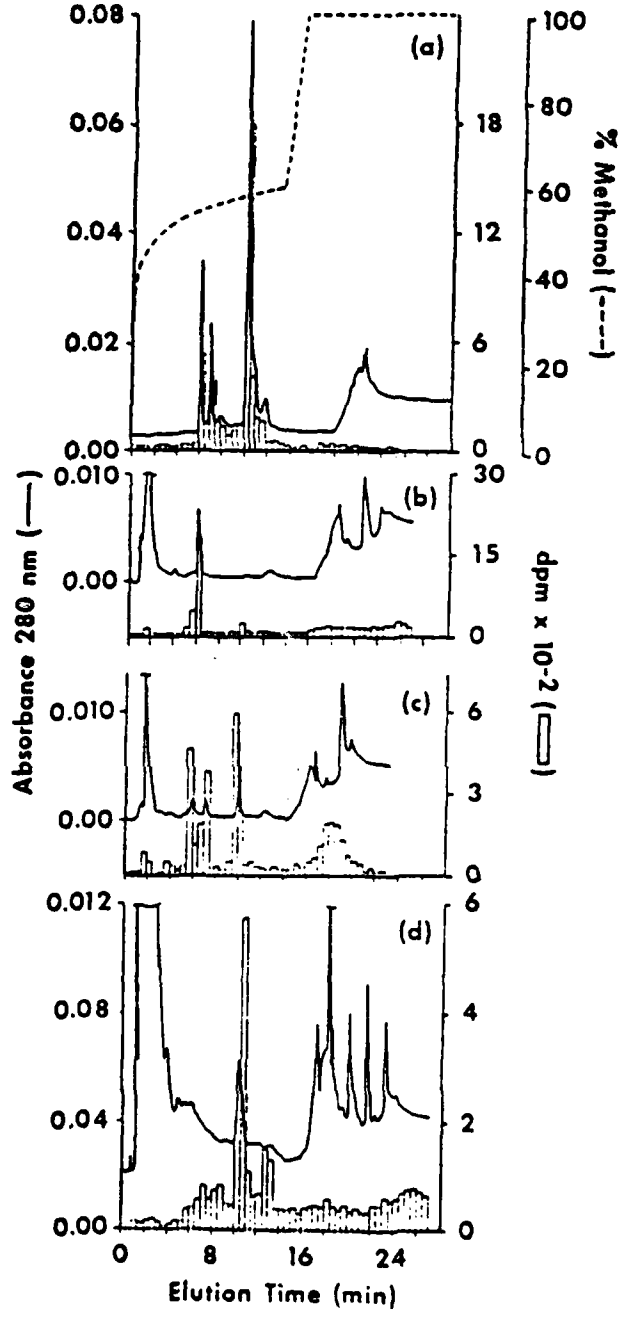


Figure 3 - Effect of pH on xanthine oxidase activity and the binding of 1-nitropyrene to DNA. The incubation contained 50 mM potassium phosphate, 3.7 mM hypoxanthine, 2 mg/ml calf thymus DNA, 0.1 U/ml xanthine oxidase and 20 μM [^{3}H]1-nitropyrene. The incubations were terminated after 1 hour and the extent of binding determined as described in Materials and Methods. The xanthine oxidase oxidation of hypoxanthine to uric acid was determined spectrophotometrically (36). The theoretical 1-nitropyrene binding curve was constructed as described in the text.



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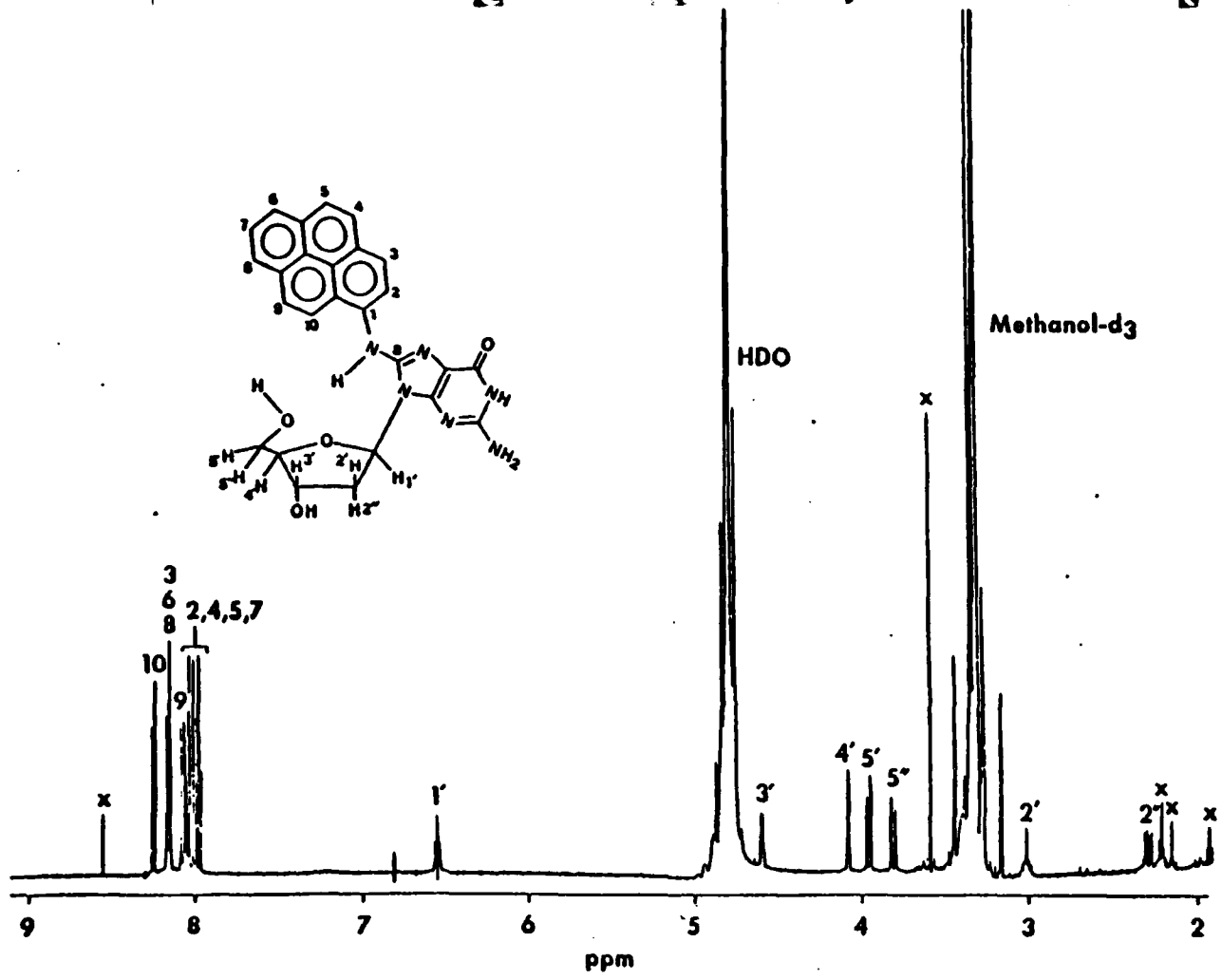

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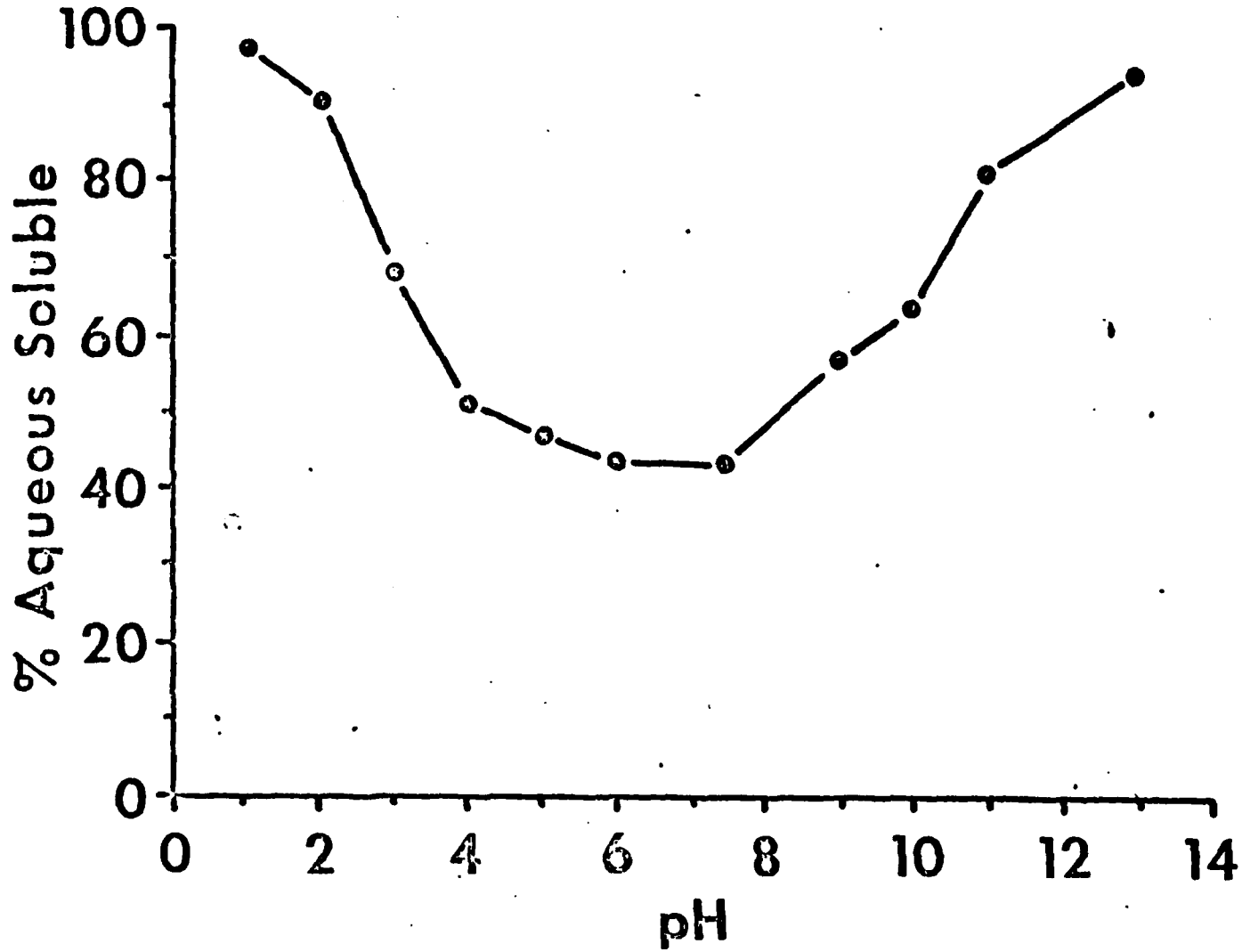

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A COMPARISON OF EXPRESSION OF NEOPLASTIC POTENTIAL OF CARCINOGEN-TRANSFORMED HUMAN FIBROBLASTS IN NUDE MICE AND IN CHICK EMBRYONIC SKIN

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SUMMARY

Human foreskin fibroblasts transformed by representative chemicals from five different classes of chemical carcinogens, some requiring enzymatic activation and direct acting carcinogens, produced cell populations that exhibited anchorage-independent growth and expression of neoplastic potential in either nude mice or chick-embryonic skin (CES). There is a high degree of correlation between tumor incidence and invasiveness of CES. The unique feature of CES is the rapidity of expression of cellular neoplasia and interpretation of the simulated tumor in 4 d as a simulated fibrosarcoma. This method represents a system that can be used to evaluate human carcinogens in vitro in 6 to 10 wk.

Key words: chemical carcinogens; neoplasia; human cell transformation.

INTRODUCTION

Recently we reported on the transformation of human cells to a stage of anchorage-independent growth (1) and neoplasia (2). Neoplasia was evaluated in nude mice. Administration was by two different routes: one by injection of transformed cells subcutaneously into the subclavicle region (1); and the other by injection of a "bolus" of cells intracranially (3). In the case of the subcutaneous route of injection 4.5 to 6 wk elapsed before we could label the take as positive. Unequivocal identification of the tumor was accomplished after excision of the tumor followed by histopathological examination requiring an additional extended period of time.

Treated cells injected intracranially into the frontal parietal sinus of the mouse killed the mice within 30 to 45 d. This has been described as "mouse lethality" (3). The procedure described here presents data on an in vitro procedure that can assay rapidly for neoplasia within 3 d, therefore eliminating the requirement of waiting 1 to 1.5 yr until the sham intracranial or subcutaneous injected control mice die of natural causes.

MATERIALS AND METHODS

Cell culture. Primary fibroblast cultures of foreskin tissue were prepared in the following manner: Human foreskin tissue was minced into 2-mm segments in minimum essential medium-Hanks' balanced salt medium (MEM-HBSS) containing 25 mm HEPES buffer at pH 7.2. The tissue was rinsed three times in this medium and the tissue fragments transferred to 20 ml of MEM-HBSS supplemented with 20% fetal bovine serum (FBS) (Sterile Systems, Logan, UT), complete growth medium (CM), and containing 0.1% collagenase (115 U mg CLS, Worthington Biochemical Corp., Freehold, NJ). After dispersion of the tissue at 37° C in a 4% CO_2-enriched air atmosphere for 6 to 8 h, the cells were recovered by centrifugation at 650 ×*g* for 7 min at 12° C. The cell pellet was washed twice with CM and the final cell suspension seeded into

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a 75 cm² flask. The cell populations were allowed to attach for 72 h and were refed once.

On Day 5 after seeding, the fibroblasts were selectively detached with 0.1% trypsin (4). The fibroblast cultures were then seeded into CM and prepared for treatment with carcinogen.

Suspension of chemical carcinogen and insulin. The treatment process required that to be effective in the treatment regimen the carcinogens be solvated in a prescribed manner. The carcinogens, either aflatoxin B_1 (AFB_1), B-propiolactone (B-P1), methylazoxymethanol acetate (MAMA), benzo(a)pyrene(BaP), benzo(a)pyrene-diol-epoxide-I (anti) [BPDE(I)], or tetrahydrodimethylbenzanthracene (TH-DMBA) were solvated in Spectrar acetone under red or gold light under argon. These stock solutions were made up fresh for each experiment. The working solutions at effective cytotoxic doses of 0, 25, or 50% were prepared from the stock solutions in complete growth medium (CM). These solutions were added immediately to the cells as described previously (1,2).

Transformation protocol. Fibroblast cell populations blocked in G_1, released from the block, and treated with the carcinogen in S phase of the cell cycle (2,5) were then serially subpassaged for 16 population doublings (PDL) before passage through soft agar.

All the carcinogens used in these experiments were added to the cell populations when the cells were in early S. The treated populations were exposed to the carcinogens for 30 min such as for BPDE(I) with a brief half-life or up to 12 h for carcinogens with a long half-life [B(a)P].

Treatment was terminated when the experimental medium was removed and the population immediately passaged at a 1-to-2 split ratio into CM containing additional growth supplements of X2 vitamins, X8 nonessential amino acids, and 20% FBS. The treated and untreated cultures at 80 to 90% saturation density were passaged at 1-to-10 split ratio into the enriched growth medium for 16 PDL.

Growth in soft agar. The carcinogen treated and untreated populations at PDL 16 were seeded at 50,000 cells/25 cm² area in 2 ml of 0.33% soft agar prepared in Dulbecco's Lo Cal medium (Biolabs, Northbrook, IL) (6,7). The cultures were incubated in a 4% carbon dioxide enriched air atmosphere and refed every 3 to 5 d. The incidence of colony formation was determined, and after 3 wk the colonies were removed and seeded into 25 cm² flasks containing CM.

These populations were then prepared for either nude mice injection or seeding on CES in vitro.

Evaluation of neoplasia. The treated populations that exhibited anchorage independent growth were evaluated in 6-wk old nude mice (Sprague-Dawley, Madison, WI). Because untreated cell populations would not passage through soft agar, companion untreated cultures that had not passaged through soft agar were evaluated in the nude mice (8) and on CES. The mice were irradiated with 450 RAD (from a ^{137}Cs source) 48 h before subcutaneous injection of 5×10^6 treated or control cell populations (6). After 6 wk the tumors were counted and the incidence of tumor formation recorded. The lack of tumor formation in injected mice was not recorded until the mice died (1 to 1.5 yr).

These same treated and untreated cell populations were seeded onto CES in vitro. The CES organ cultures were modified in the following manner to optimize the frequency of success for a rapid evaluation of cellular neoplasia (9). We (10) have successfully applied this technique to evaluation of carcinogen induced epithelial cell neoplasia. Growth of the treated epithelial cells into the CES simulated squamous cell carcinomas.

Fertile eggs were incubated 9 to 10 d in a humidified egg incubator (Humidair Incubator Co., New Madison, OH). The embryos were removed and the skin removed from the embryo. These pieces of skin were then placed on agar bases containing 10 parts of 1% agar (Bacto-agar) in Earle's balanced salt solution without bicarbonate. This preparation also contained four parts FBS and four parts chick embryo extract (9). The enriched agar base was poured over a steel grid implanted into the agar base. A 6 to 8 mm d section of skin was layered onto the base dermis side up. A sterile glass ring 2 mm in thickness and 8 mm d was laid over the skin. Populations from untreated and treated cell populations, containing 10^6 cells, were suspended in 0.025 ml of CM+20% FBS and seeded into the rings. The dishes were then incubated in a 4% CO_2 humidified enriched air atmosphere at 37° C. On Day 2 the system was fixed in Bouin's solution, embedded in paraffin stained with hematoxylin and eosin, and evaluated by light microscopy.

RESULTS

There is variation in cytotoxic response of treated fibroblast to the treatment when the fibro-

blasts are obtained from different donors. The values reported in Table 1 for ED_{50} values are mean values for eight different wells. These concentrations were used as a carcinogenic dose for treatment of the populations in S. The compounds BPDE(I), B-propiolactone, and MAMA have a relative brief half-life and were left on the cells in S for 30 min for BPDE(I) or 3 h for B-PL or MAMA. The chemicals, either Aflatoxin B_1 or TH-DMBA, were left on the cells for 12 h.

These cell populations treated in S were serially passaged for 16 PDL prior to seeding the populations into soft agar (Table 1). All of the populations treated with each carcinogen exhibited colony formation when seeded into soft agar.

Once the protocol for transformation had been completed and abnormal colonies identified the cells were serially passaged. These cultures were continually passaged for another 16 PDL. At that time the populations were seeded into soft agar. We have observed formation from 10 to 900 colonies/10^5 cells (Table 1) depending upon the carcinogen treatment. Aflatoxin B_1 transformed cells produced 10 colonies/10^6 treated cells seeded in soft agar whereas methylazoxymethanol acetate treated cells produced 900 cells/10^6 treated cells. These colonies were removed, pooled, and reseeded in 25 cm^2 flask. After 10 PDL, 5×10^6 cells were injected subcutaneously into nude mice. Tumor takes were counted 4 wk later. In three out of five transformed cell populations exhibiting anchorage independent growth in soft agar, all three formed tumors in nude mice (Table 1). Tumor incidence ranges from two mice giving rise to undifferentiated mesenchymal tumors, after the reception of 5×10^6 methylazoxymethanol

TABLE 1

ANCHORAGE INDEPENDENT GROWTH, TUMOR FORMATION IN NUDE MICE, AND CELLULAR NEOPLASIA OF CARCINOGEN TREATED HUMAN FORESKIN FIBROBLASTS IN VITRO

Compound[a]	Concentration[b]	Frequency Colony Growth in Soft Agar[c]	Tumor Incidence[d]	Incidence of Invasiveness CES[e]
	μg/ml			
Aflatoxin B_1	10	10	8/14	6/6
β-propiolactone	13	14	3/4	6/6
Benzo(a)pyrene-diol-epoxide-I	0.1	26	ND	6/6
Tetrahydro-7,12 dimethylbenz(a)anthracene	1.0	84	ND	6/6
Methylazoxymethanol acetate	3.6	900	2/16	6/6

[a] The selection of these compounds was based on prior knowledge that they represent a cross section of compounds requiring no activation. The compounds aflatoxin B_1, benzo(a)pyrene-diol-epoxide-I were furnished by the National Cancer Institute's (NCI) chemical repository I.I.T.R.I.; B-propiolactone was a gift from Dr. Joseph DiPaolo, DCCP, National Cancer Institute, Bethesda, MD. Methylazoxymethanol acetate was a gift from Ms. Marilyn George, Department of Defense, AFSOR, Wright Patterson Airforce Base, Dayton, OH; 1,2,3,4 tetrahydro-7,12 dimethylbenz(a)anthracene was synthesized and characterized here at O.S.U. by Dr. Donald Witiak et al. This compound is the reduced A-ring analogue of 7,12 dimethylbenz(a)anthracene (7,12 DMBA).

[b] These concentrations were selected because of their individual effects on cell proliferation as measured by alternation in cloning of cell populations at a low cell density of 1,000 cells/25 cm^2 (6). The concentrations used here represent 50% cytotoxic doses.

[c] Frequency of colony growth in soft agar is described as the formation of colonies of 50 cells or more when 50,000 cells at 20 PDL were seeded into 2 ml of 0.33% agar supplemented with LoCal and 20% FBS, overlaid over a 2.0% agar base supplemented with RPMI 1629 and 20% FBS in a 25 cm^2 well. The colonies were counted after 28 d. The frequency of growth is expressed as the number of colonies formed per 10^4 cells.

[d] The tumor incidence is expressed as follows: The numerator value is the number of mice giving rise to tumors 0.8 to 1.2 cm in size at 4 to 6 wk after injection of 5×10^6 cells per animal subcutaneously, and the denominator is the total number of nude mice injected. All animals used in these experiments were 5 to 6 wk of age and preirradiated with 450 RADS whole body irradiation 24 to 48 h before injection. All animals were housed under cyclic light-dark cycle over a 12-12 h cycle. The lighting in the animal room was a GE gold fluorescent light.

[e] The incidence of invasiveness of CES in vitro was measured on the 4th d, i.e. 3 full d after seeding of the cells on CES. Treated cell populations that exhibited anchorage-independent growth and untreated populations were examined on CES. The incidence of invasiveness is expressed as follows: The numerator value is the number of CES positive for the presence of invading human transformed cells seeded per total number of CES seeded. These figures represent values for three to four replicates. Out of three to four slides per CES we observed that at least one out of three slides was positive for all populations examined.

ND = not done.

acetate treated cells isolated from soft agar per 16 mice receiving the inoculum, to 8/14 receiving the aflatoxin treated cells. The nude mice receiving treated cells were not scored as negative until

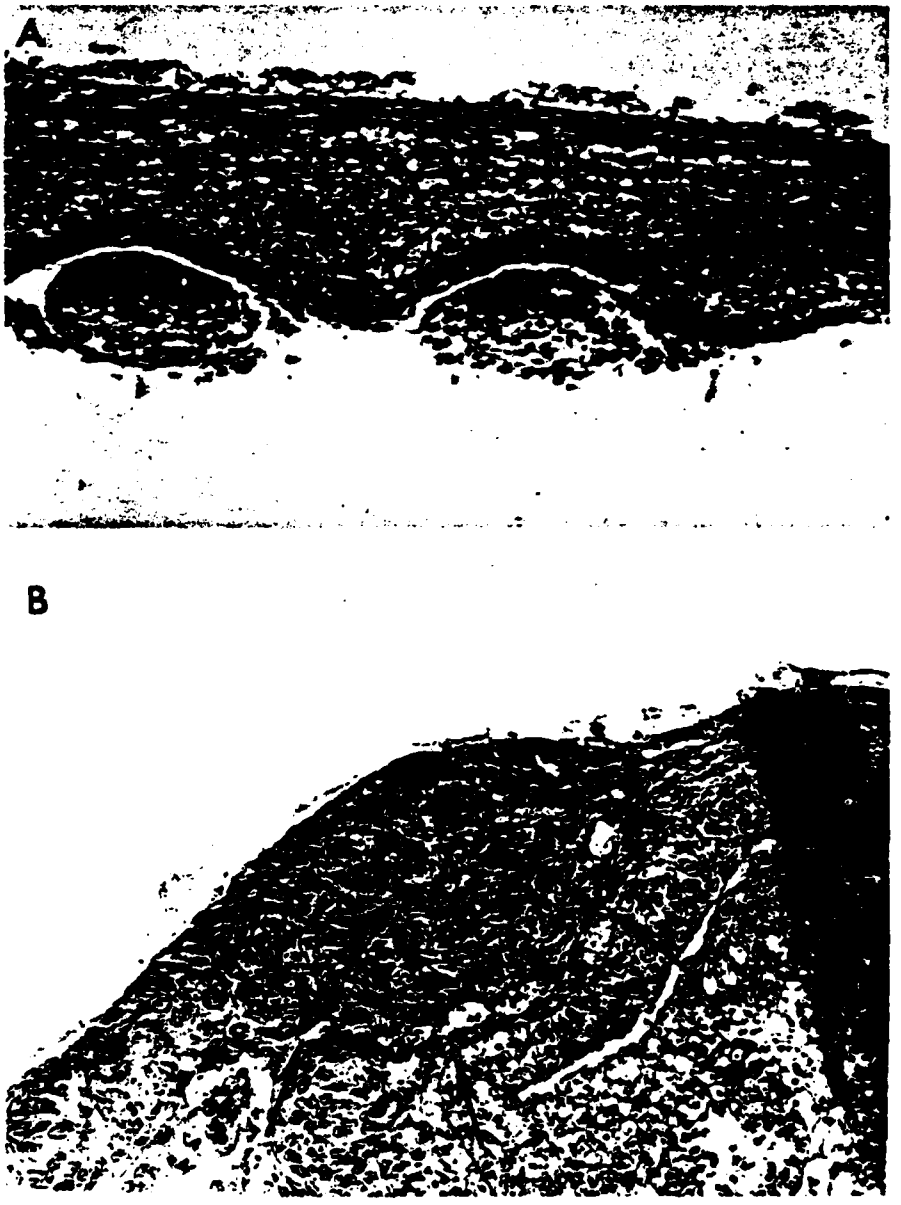

FIG. 1. *A*. Chick embryonic (CES from 9-d-old chick embryo) prepared as described in Materials and Methods section were supplanted onto a 5 ml 1% Bacto-agar base supplemented with growth factors. One hundred thousand untreated cells were seeded onto the CES and these organ cultures incubated at 37° C for 4 d in a 4% CO_2 enriched air environment. ×100. These fibroblasts appeared as necrotic cells on the dermal surface of the CES. *B*. CES organ cultures prepared as described above received 10^4 cells of transformed cells re-established from 0.33% agar layers. After 4 d in a 4% CO_2 enriched air environment they were evaluated. Invasion of the CES by the transformed cells is illustrated by *arrows*.

1 yr later. The nude mice under our environmental conditions lived for 1 to 1.5 yr. The same untreated (Fig. 1*A*) and treated (Fig. 1*B*) populations that were injected into the nude mice were seeded on the CES. After 3 d the tissues were fixed and stained with hematoxylin and eosin; then five micron thick sections were examined (Fig. 1*B*). The transformed cells penetrated into the layers of the CES, and in many cases complete interruption of 8 to 10 layers of chick cells were observed. The presence of mitotic figures in the invading transformed cell populations was observed. The invasive features of the tumors were determined to be compatible with a simulated fibrosarcoma. The invasiveness of these proliferating transformed human fibroblast populations implies malignancy but is not to be compared to metastases (Fig. 1*B*). We want to imply that these chemical carcinogen transformed cells contain the ability to form localized growth (tumors) on CES. We state also that there is a strong correlation between the tumor incidence in nude mice, growth in soft agar, and microtumor formation in CES. Untreated cell populations seeded onto CES did not penetrate the upper layer of CES (Fig. 1*A*). Moreover, transformed human cells isolated from a squamous cell carcinoma in the nasopharyngeal area and testicular carcinoma cells formed localized tumors in nude mice and invaded CES. The chemically transformed populations from all five carcinogen treated populations (Table 1) were designated as fibrosarcomas.

Discussion

In recent investigations we have been able to evaluate transformed human cell populations for their anchorage-independent growth in a semi-solid medium (1) or for their tumor potential in nude mice (2). Moreover, we noted that in each case the tumors produced by chemical carcinogen transformed cell populations that exhibited anchorage-independent growth characteristics were classified as undifferentiated mesenchymal tumors (1,2) when evaluated in nude mice, whereas FeSV transformed cell populations induced tumors were classified as fibromas (11), and physical carcinogen (UV_{254nm} or ^{137}Cs) induced transformed cell populations (7) were classified as myxofibromas. These evaluations of neoplasia in the nude mouse took several weeks to one year (1,2,6). With CES the evaluations of cellular neoplasia can be completed within 4 d after seeding of the cells onto the dermis of the CES. We have not observed invasion of the CES by untreated cell populations. In every CES that received human squamous cell carcinoma cells from the nasopharyngeal area or testicular carcinoma cells, when tested they always formed tumors in nude mice and invaded the CES. With both negative and positive control populations we concluded that the CES system can be used effectively in vitro to evaluate carcinogen induced neoplasia of diploid human cells to a neoplastic stage.

With the current technology to transform human cells in vitro with physical and chemical carcinogens we can now evaluate the neoplastic potential of these cells on CES in vitro with a high degree of reliability and reproducibility and within a reasonable period of time from induction to neoplasia of 6 to 10 wk instead of 1 to 1.5 yr. Recently we adapted this assay to evaluating chemically transformed human epithelial foreskin populations in vitro for evidence of cellular neoplasia. The system lends itself to a rapid, inexpensive assay that has a high correlation with growth of the treated cells in soft agar and growth of transformed cells in nude mice.

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Benzo[a]pyrene diol epoxide DNA adduct formation in transformable and non-transformable human foreskin fibroblast cells *in vitro*

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Abstract

The uptake of benzo[a]pyrene (BP) by low passage (LP) and high passage (HP) human skin fibroblast cells is followed by its transport into the nucleus as the parent compound. When the LP and HP cells were treated with BP for 24 h and the DNA was isolated and enzymatically digested, several DNA adducts were detected. In both the LP and HP cells a small amount of the radiolabel was associated with the 7β-BPDE-I – dG, 7α-BPDE-I – dG and BPDE-II – dG adducts. Although there were no major qualitative differences in the adducts formed in the LP and HP cells, a higher proportion of the radiolabel was associated with the 7β-BPDE-I – dG adduct in the LP cells. When LP or HP cells were treated with BPDE-I, the ultimate carcinogenic form of BP, similar levels of DNA modification were observed in the two cell types and the h.p.l.c. profiles of these adducts were essentially identical. BPDE-I induced a carcinogenic event in the LP but not the HP cells as measured by anchorage independent growth in soft agar and cellular invasiveness of the chick embryonic skin organ cultures.

Introduction

Several studies have strongly implicated 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (7β-BPDE-I)* as the major metabolic intermediate involved in the covalent binding of benzo[a]pyrene (BP) to nucleic acids (1,2,4,5). The major RNA and DNA adducts present in human cells exposed to BP have been shown to be generated by 9/10 *trans*-addition of guanine, at the 2-amino group, to 7β-BPDE-I (6). Lesser amounts of N^2-substituted deoxyguanosine adducts derived from 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE-II) have also been detected (7,8). Although guanine residues are the major nucleic acid targets, BPDE-I has also been shown to form minor adducts with adenine and cytosine residues (9,10).

Previous studies of Milo, *et al.* have indicated that although BP can induce DNA damage in logarithmically growing low passage (LP) and high passage (HP) cells (11,12), only the LP cells can be neoplastically transformed by this polynuclear hydrocarbon (PNH) (Milo, *et al.*; unpublished data). When treated with BP, these cells exhibit morphological changes, anchorage independent growth and tumor production in athymic nude mice (16) or on the chick embryonic skin (CES) organ cultures (13).

When LP and HP human skin fibroblast cells are treated with BP, the PNH first accumulates in the cytoplasm where it is associated with different cytoplasmic lipoprotein complexes (14,15). The PNH is then transported into the nucleus within 24 h (16). H.p.l.c. analysis of the BP metabolites non-covalently associated with the cellular macromolecules has indicated that a major portion of the BP initially bound to the cytoplasmic lipoprotein complex is transported to the nucleus as the parent compound (14,17).

The present studies were undertaken to determine the nature of the BP metabolites covalently bound to DNA, isolated from treated LP and HP human skin fibroblast cells. It was also important to examine the induction of a carcinogenic event in the LP and HP cells by BPDE-I.

Materials and Methods

Chemicals

Generally labeled [G-^{3}H]BP (37 Ci/mmol) was diluted with unlabeled BP (50 μg/ml) to a specific activity of 19 Ci/mmol. All BP dilutions were carried out under gold light (F40G bulb, G.E.) in an atmosphere of argon and the stock solution was stored at −20°C. The [G-^{3}H]BP sample was >95% pure as determined by h.p.l.c. BPDE-I and [^{3}H]-BPDE-I (398-565 mCi/mmol) were obtained from the National Cancer Institute Repository.

Cell cultures

Primary cultures of human neonatal foreskin (HNF) fibroblast cells were established as described previously (18). HNF cells at population doubling (PDL) 1, growing on three 75 sq cm tissue culture flasks, were trypsinized, pooled, and seeded into 150 sq cm tissue culture dishes. The cells were serially passaged and maintained in complete growth medium (CM) composed of Eagle's minimum essential medium – 25 mM Hepes buffer (pH 7.2) supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM glutamine, 50 μg/ml gentocin, 0.2% sodium bicarbonate and 10% fetal bovine serum (FBS). Sixty tissue culture dishes of preconfluent HNF cells between PDL 6 – 25 (derived from the same tissue sample) were incubated with 0.112 μM [G-^{3}H]BP (19 Ci/mmol) for 24 h in CM or 0.114 μM [^{3}H]BPDE-I for 3 h in CM without FBS (15).

Carcinogen treatment and neoplastic transformation

Actively growing preconfluent HNF cell monolayers between PDL 4 to 5 were blocked at the G_1 phase of the cell cycle by seeding cultures with Dulbecco's modified Eagle's medium without arginine and glutamine, supplemented with 50 μg/ml gentocin, 1 mM sodium pyruvate and 10% dialyzed FBS (20). The flasks were incubated at 37°C in a 4% CO_2-enriched air atmosphere. Twenty four hours after seeding, the medium was removed and the cells were refed with CM supplemented with 0.5 U/ml insulin.

The synchronized monolayers, in S phase of the cell cycle, were treated with BPDE-I by the procedure of Heflich, *et al.* (21). Thirty six hours after seeding, the CM was decanted and the cell cultures were refed with CM without FBS supplemented with 0.5 U/ml insulin. The flasks were treated

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*Abbreviations: 7β-BPDE-I, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; 7α-BPDE-I, 7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; BP, benzo[a]pyrene; BPDE-II, 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; LP, low passage; HP, high passage; PNH, polynuclear hydrocarbon; CES, chick embryonic skin; HNF, human neonatal foreskin; PDL, population doubling; CM, complete growth medium; FBS, fetal bovine serum; BPDE-I – dG, is the deoxyguanosine adduct with 7β-BPDE-I or 7α-BPDE-I.

[1]ED 25 – dose effective in reducing the cloning efficiency to 25% of the control.

[2]ED 50 – dose effective in reducing the cloning efficiency to 50% of the control.

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with 0.057 μM (ED 25[1] dose) and 0.114 μM (ED 50[2] dose) of BPDE-I in acetone. Monolayers to which an equal volume of acetone was added served as controls. Three hours after treatment, the medium containing BPDE-I was removed and the cell cultures were refed with CM supplemented with 0.5 U/ml insulin.

Forty eight hours after seeding, the treated and control cell cultures were passaged at a 1:2 split ratio into CM supplemented with 2 x essential vitamins, 8 x non-essential amino acids and 20% FBS (selection medium). The cell populations were serially passaged at a 1:10 split ratio into the selection medium. After 16 PDL, the cell populations were seeded into soft agar as previously described (12,20). The soft agar cultures were incubated at 37°C in a 4% CO_2-enriched air atmosphere. Three weeks later, the colonies were removed and seeded into the selection medium. The cell cultures were serially passaged and maintained in the selection medium until they were seeded on the CES to assay for cellular neoplasia (22).

Analysis of DNA adducts.

The nuclear fraction was prepared by a modification of the procedure of Chauveau, *et al.* (16,19). The nuclear pellet, suspended in 0.5 ml of 10 mM Tris-HCl – 1 mM Na_2EDTA buffer (pH 7.5) containing 0.1% SDS, was immediately frozen under liquid nitrogen and stored at −20°C.

The nuclear suspension was diluted to 2 – 5 ml with 10 mM Tris-HCl, pH 7.0 containing 0.15 M NaCl and 1% SDS and was extracted several times (3 to 6) with water saturated, redistilled phenol containing 0.1% 8-hydroxyquinoline. The DNA was then precipitated by addition of 0.2 M sodium acetate, pH 5.0 and two volumes of ethanol at −25°C overnight. The DNA was redissolved in water and precipitated with ethanol in presence of sodium acetate, until at least 95% of the radioactivity was precipitable. The DNA (< 1 mg/ml) was dissolved in 10 mM Tris-HCl – 0.1 M NaCl – 5 mM $MgCl_2$ (pH 7.9) and was treated consecutively with 200 units of DNase I for 4 h, 5 units of alkaline phosphatase for 2 h, 2 units of phosphodiesterase I for 4 h, 2 units of phosphodiesterase II for 4 h and 5 units of alkaline phosphatase for 4 h.

The resulting deoxyribonucleosides were then chromatographed on a Sephadex LH-20 column. After application of the sample, the column was washed with water to remove unmodified deoxyribonucleosides. The modified adducts were eluted with 80% methanol and the solvent was evaporated under reduced pressure. The sample was dissolved in 10 μl of methanol containing unlabeled BPDE-I – DNA adducts (6,9) and was analyzed by h.p.l.c. The sample was separated on a Dupont Instruments Model 830 or 850 High Pressure Liquid Chromatograph fitted with a C_{18} μ-Bondapak column (Waters Associates) using a concave gradient number 2 from 30 – 60% methanol in water at 50°C and a flow rate of 1 ml/minute (for Model 850) or at 750 psi (for Model 830). Samples were collected and the radioactivity was assayed. In some instances the samples were acetylated for reanalysis by h.p.l.c. as previously described (6).

Results

The LP and HP human skin fibroblast cells were treated with [G-^{3}H]BP and the cellular DNA isolated. The level of modification of the DNA was variable, 4.2 ± 4.7 adducts/10^8 bases for LP cells and 7.5 ± 4.9 adducts/10^8 bases for HP cells, but did not appear closely related to the ability of these cells to be transformed by BP. The LP cells yielded 86 transformants (colonies in soft agar) per 100 000 cells for cells containing 4.2 ± 4.7 adducts/10^8 bases. When the HP cells were treated with BP (7.5 ± 4.9 adducts/10^8 bases), no colonies in soft agar were observed. The isolated DNA was digested to release the modified deoxyribonucleosides which were separated by chromatography on columns of Sephadex LH-20. Between 60 and 75% of the radioactivity was not retained by the Sephadex LH-20 columns. The modified deoxyribonucleosides were then analyzed by h.p.l.c. using appropriate markers synthesized *in vitro* (6,9). The profiles of the DNA adducts isolated from the LP and HP cells are shown in Figures 1 and 2, respectively. The first peak to elute cochromatographed with a hydrolysis product of 7β-BPDE-I. However, when this material was collected, acetylated and reanalyzed by h.p.l.c. (6), the radioactivity (60 min) and the tetrol (68 min) were clearly resolved. The major radioactive component corresponding to the second peak did not elute with any of our standard compounds. Components were also detected from both cell types which co-eluted with the 7α-BPDE-I – dG, 7β-BPDE-I – dG and BPDE-II – dG adducts. The 7β-BPDE-I – dG adduct, present in higher proportions in the LP cells compared to the HP cells (29% ± 6.2 in LP cells and 8.3% ± 2.9 in HP cells) was acetylated and reanalyzed by h.p.l.c. The radioactivity and synthetic standards co-eluted (76 min) confirming their identity. Little radioactivity was detected in the region where deoxyadenosine adducts elute.

When LP and HP cells derived from the same tissue sample were treated with [^{3}H]BPDE-I, similar levels of DNA modification were observed (2.1 and 3.5 adducts/10^8 bases, respectively). Digestion of the modified DNA samples and separation by h.p.l.c. (Figure 3) showed that at least 80% of the radioactivity could be accounted for by two adducts, the major one being the 7β-BPDE-I – dG (39% in LP cells and 41% in HP cells) and the minor one being the 7α-BPDE-I – dG diastereoisomer (34% in LP cells and 23% in HP cells).

PNH treatment of the LP and HP human cells in culture was carried out by the procedure of Milo and DiPaolo (12,20). Randomly proliferating LP and HP cell populations blocked at the G_1 phase of the cell cycle for 24 h, were treated

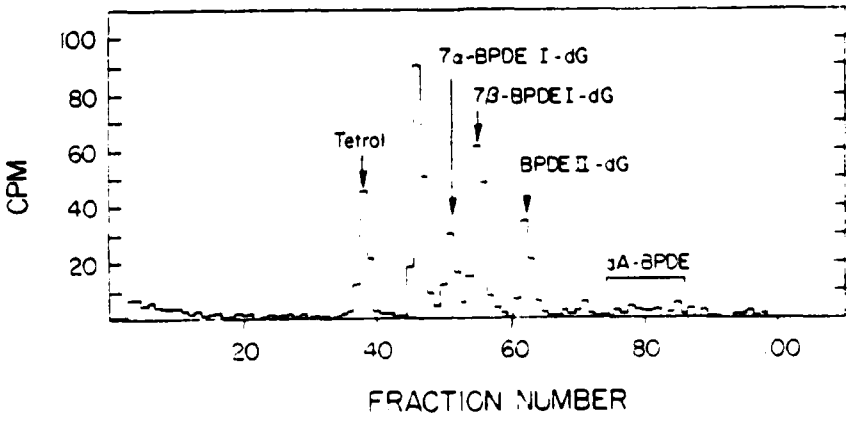

Fig. 1. H.p.l.c. profile of DNA adducts formed by LP HNF cells incubated with [G-^{3}H]BP for 24 h. PDL 6 cells were treated with [G-^{3}H]BP for 24 h and the DNA was isolated and enzymatically digested. The modified deoxynucleosides, separated by Sephadex LH-20 chromatography, were cochromatographed on h.p.l.c. with authentic reference standards.

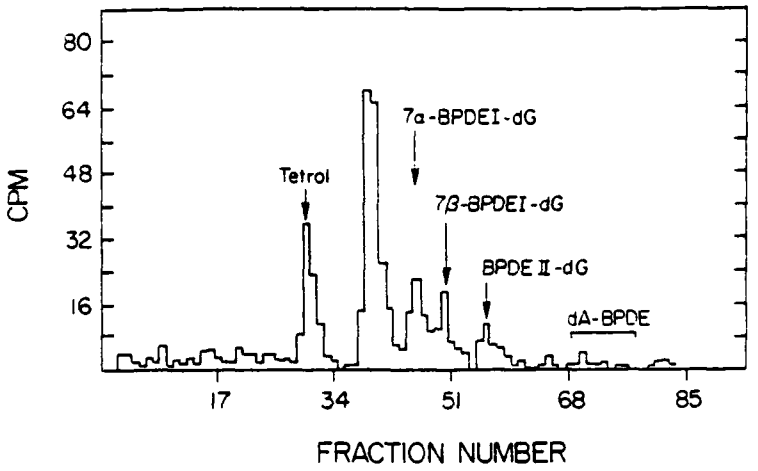

Fig. 2. H.p.l.c. profile of DNA adducts formed by HP HNF cells incubated with [G-^{3}H]BP for 24 h. PDL 25 cells were treated with [G-^{3}H]BP for 24 h and the DNA adducts were analyzed by h.p.l.c. as described in Figure 1.

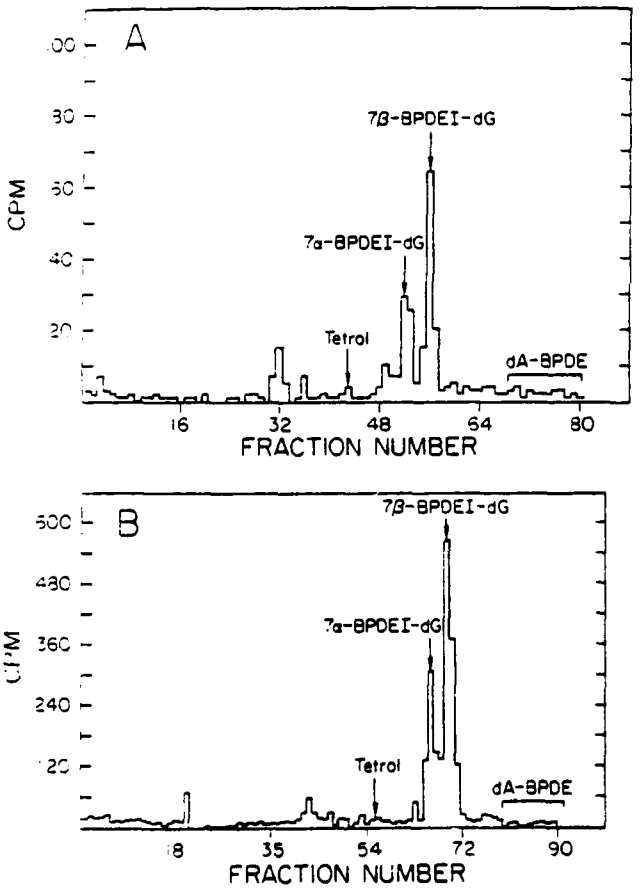

Fig. 3. H.p.l.c. profile of DNA adducts from [G-^{3}H]BPDE-I-treated HNF cells. (A) LP-PDL6 cells and (B) HP-PDL 25 cells were treated with ^{3}H-BPDE-I for 3 h and the DNA adducts analyzed by h.p.l.c. (Figure 1).

with insulin to release them from the block. Twelve hours later (2 h into the S phase of the cell cycle) the cells were treated for 3 h with BPDE-I in serum free medium. The LP and HP cell populations were serially passaged for 16 PDL and then seeded into soft agar. In the LP cells treated with BPDE-I, colonies were observed 10 to 12 days following seeding of the single cell suspension in soft agar (Figure 4). Each colony contained 30 – 50 cells. Although the control

Fig. 4. *Growth of BPDE-I treated LP cells in soft agar.* LP cell populations treated with BPDE-I as described under **Materials and Methods** were seeded at 50 000 cells/25 cm^2 wells into soft agar. The soft agar cultures were incubated at 37°C in a 4% CO_2-enriched air atmosphere for 3 weeks.

Table I. Anchorage independent growth and cellular invasiveness of BPDE-I[a] treated cell populations.

Experiment no.	Passage	Concentration (μM)	No. of colonies/ 10^5 cells seeded in soft agar	Frequency of invasiveness on CES (15)
1	LP	0.114	26	6/6
		0.057	10	
2	LP	0.114	33	6/6
		0.057	15	
3	HP	0.114	zero	N.D.[b]
		0.114	zero	N.D.
		0.057	zero	N.D.
4	HP	0.114	zero	N.D.
		0.114	zero	N.D.
		0.057	zero	N.D.

[a]Treatment of the LP cell populations with BP and the transformation of these cells has been previously reported (13,16). Both the frequency of colony formation in soft agar and incidence of cellular invasiveness have been reported (13).[b]N.D., not determined. It has been our experience that treated populations that do not exhibit anchorage independent growth do not invade CES (22).

cultures did not form any colonies, the frequency of colony formation of cells treated with 0.057 μM and 0.114 μM BPDE-I were 10 – 15 and 26 – 33 per 10^5 cells seeded in soft agar (Table I). In contrast, when HP cells treated with 0.057 μM or 0.114 μM BPDE-I were seeded in soft agar, no colony formation was observed even after four weeks. Moreover, treatment of the HP cells with various other carcinogens also failed to induce neoplastic transformation (Milo, *et al.*, unpublished data).

Three weeks after seeding into soft agar, the colonies derived from the LP cells were removed and the cells passaged in the selection medium. The transformed cell populations exhibiting anchorage independence were checked for cellular neoplasia on the CES organ cultures. The BPDE-I treated LP cells exhibited invasiveness on the chick skins while the untreated cells were not invasive (Figure 5). Histopathology of the invaded chick skins was interpreted as a fibrosarcoma.

Discussion

Various workers have shown that PNH's, such as BP, can be metabolically activated to bay region diol-epoxides which in turn may react with cellular macromolecules, induce mutations and transformation in various cell types and induce tumors in animals (1 – 6,23). However, the induction of a

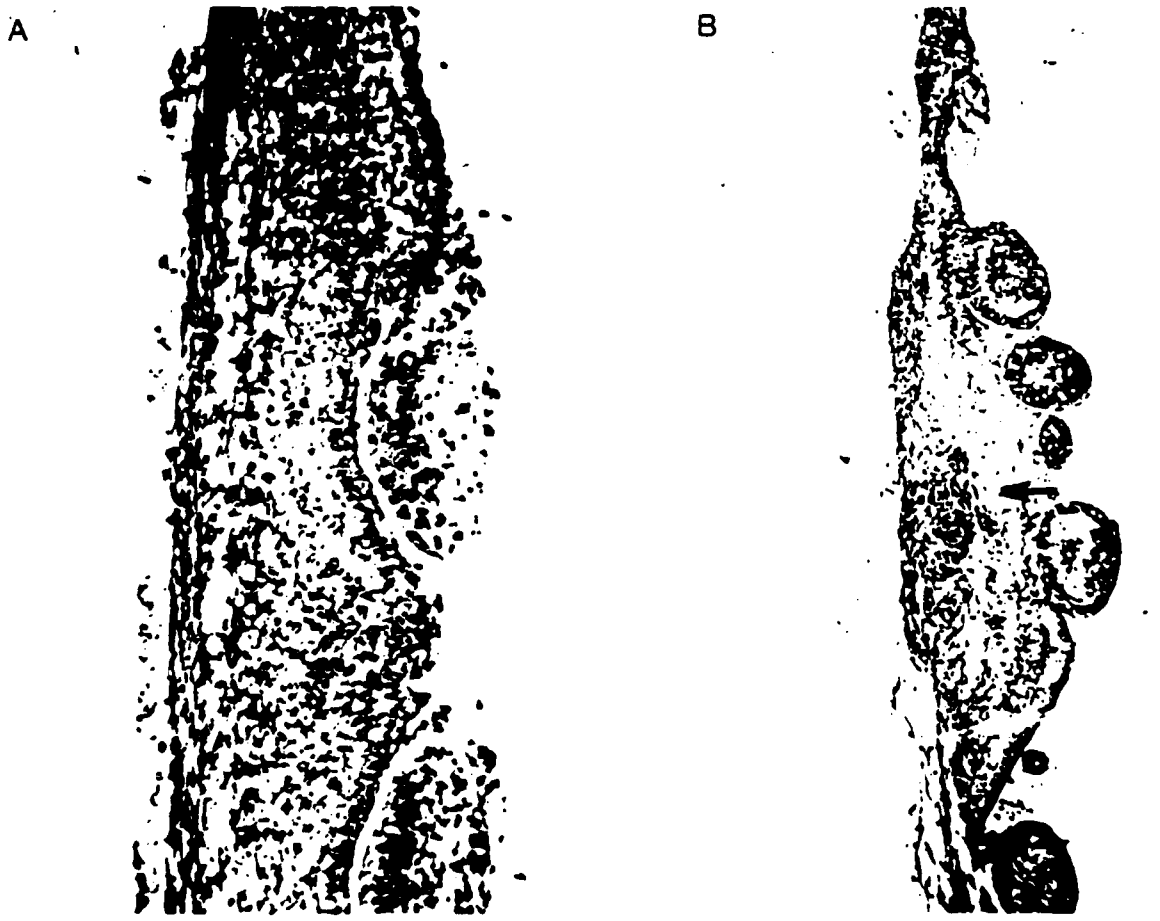

Fig. 5. Invasiveness of BPDE-I treated cell populations on CES. LP cells exhibiting anchorage independent growth were removed serially passaged and seeded on the CES (22). Three days later, the CES were fixed in Bouins Solution, stained with hematoxylin and eosin and microscopically examined. (A) represents normal *untreated* fibroblast cells seeded on the CES at 400 x magnification. (B) represents BPDE treated cell populations growing on the CES at 200 x magnification.

tumor in an animal as a result of exposure to PNH's is a multistep process. The availability of cells derived from a single source – human foreskin, which either have or lack the ability to be transformed by BP – has enabled us to investigate some of the steps controlling and limiting this transformation.

Previous reports from our laboratory have indicated that when HNF cells (LP and HP) are treated with BP for 24 h and the metabolites are analyzed by h.p.l.c., a major portion of the PNH (90%) bound to a cytoplasmic lipoprotein complex is transported to the nucleus as unmetabolized BP (14,17).

In the present study, when either transformable or non-transformable cells were treated with [G-^{3}H]BP, a small percentage of the hydrocarbon was bound to the cellular DNA. Samples of this DNA were purified, hydrolyzed and the modified bases separated first by Sephadex LH-20 column chromatography and then by h.p.l.c. Several adducts were detected in the LP and HP cells and the profiles appeared qualitatively similar (Figures 1 and 2). The first radioactive component to elute cochromatographed with a *cis* hydrolysis product of 7,8-BPDE-I. However, when these fractions were collected and acetylated prior to reanalysis by h.p.l.c., they were shown to be clearly different. The second radioactive component eluted in the same region as an adduct prepared from the microsomal incubation of [^{3}H]9-hydroxy-BP and DNA (24). However, this adduct, described as a 9-hydroxy-4,5-oxide derivative, has never been fully characterized or prepared by direct synthesis such that adequate amounts could be obtained for complete characterization. The third component to elute cochromatographed with the 7α-BPDE-I – dG adduct. This was followed by the 7β-BPDE-I – dG adduct which has been observed in various human systems as one of the major DNA adducts (1,3,4,6). In the case of the LP cells, the fractions co-eluting with this adduct were collected, acetylated and reanalyzed by h.p.l.c. This confirmed the identity of the metabolic derivative. Radioactivity also eluted in the region of a BPDE-II – dG adduct but its identity was not confirmed by derivatization. Little material eluted in the region of the BPDE – dA adducts. There were no significant differences in the adducts formed in the LP and HP HNF cells. BPDE-I, the ultimate carcinogenic form of BP also induced neoplastic transformation in the LP but not the HP HNF cells. In addition, when LP or HP cells were treated with [^{3}H]BPDE-I, the extent of modification was similar and the 7,8-BPDE-I – dG derivative was the major DNA adduct formed. Recently, Zimmerman and Little (25) suggested that persistence of DNA adducts may account for the fixation of a carcinogenic event, however, over the time of the BP treatment of 24 h we observed little change in binding of BP to DNA in either LP or HP treated cells. They also suggested that enhancement of the transformed event occurs when cells are deprived of specific amino acids immediately prior to S. This may be however, neither LP nor HP cells showed a qualitative difference in specific adduct profile of either BP or BPDE-I treated cells. Furthermore, there was no statistical significant difference in binding of the radiolabel to the DNA of HP or LP cells. Finally, we observe no transformants in the BP-treated HP populations. This suggested that factors other than DNA adduct formation are significant in the expression of transformation of these treated cells.

Acknowledgements

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KINETICS OF MOVEMENT OF BENZO[A]PYRENE INTO TRANSFORMABLE AND NON-TRANSFORMABLE HUMAN DIPLOID FIBROBLASTS

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INTRODUCTION

Polynuclear hydrocarbons (PNH) like benzo[a]pyrene (BP) are environmental pollutants which exert their carcinogenic effect by covalent binding to cellular macromolecules (14). This binding of BP is preceded by it's activation to a BP-7,8-dihydrodiol-9,10 epoxide (anti) which has been shown to be the ultimate carcinogen (1,9,11,15). The microsomal aryl hydrocarbon hydroxylase (AHH) has been implicated in the activation of BP (3,5). Other reports also have indicated the presence of AHH activity associated with the nuclear envelope in addition to the microsomal AHH activity (6,13).

Earlier reports from our laboratory have shown that significant uptake of BP in the cytoplasm of human neonatal foreskin (HNF) cells in culture is observed at 12 hours; under these conditions, BP has been shown to bind to a cytoplasmic protein complex (12). Subsequently, optimum accumulation in the nucleus is observed at 24 hours. Although BP has been shown to damage DNA in logarithimically growing low passage (<PDL 6) and high passage (>PDL 20) cells (7), only the low passage cells can be neoplastically transformed by the PNH (4,8).

This report presents comparison of the BP metabolism in the extracellular medium, cytoplasm and nucleus of transformable and non-transformable HNF cells, 24 to 96 hours following initiation of BP treatment.

MATERIALS AND METHODS

Chemicals

Generally labelled [G-^{3}H]BP (37 Ci/mmole) was purchased from Amersham/Searle (Arlington Heights, IL.) and was diluted with unlabelled BP (50 ug/ml) to a specific

activity of 19 Ci/mmole. All BP dilutions were carrried out under yellow light in an atmosphere of argon and the stock solution was stored at -20°C. The [G-^{3}H]BP sample was >95% pure as determined by HPLC.

Cell Culture and Treatment

HNF cells between population doubling (PDL) 5 to 28 were grown and serially subpassaged as described earlier (4). Forty-eight to 72 hours after seeding, the growth medium was replaced with medium containing 0.105 µM [G-^{3}H]BP (19 Ci/mmole). After 12 hours of treatment, the cells were rinsed 6 times with incomplete medium (Eagle's Minimum Essential Medium without fetal bovine serum) and replaced with carcinogen supplemented medium containing unlabelled BP (13 µM) for up to 96 hours.

Extraction of Extracellular, Cytoplasmic and Nuclear Metabolites

All extraction procedures were carried out under yellow light and argon to minimize autoxidation. At 24 hours, 48 hours, 72 hours and 96 hours following initiation of [G-^{3}H]BP treatment, the cells were harvested. The cytoplasmic and nuclear fractions were prepared as described earlier (12). The extracellular medium samples were also removed at these time intervals.

The extracellular medium, cytoplasmic and nuclear fractions were extracted with 3 volumes of ethyl acetate in the presence of 0.8 mg/ml BHT. The organic phase was passed over anhydrous Na_2SO_4, filtered, dried under argon and stored at -20°C.

High Performance Liquid Chromatography (HPLC) Analysis

The samples were dissolved in 0.4ml methanol (Spectrar grade, Mallinckrodt, Inc., St. Louis, MO) and centrifuged at 12,000xg for 2 minutes (Eppendorf Model 5412 microcentrifuge) to remove particulate matter. Twenty microliter samples were analyzed by HPLC with a Beckman Model 322MP Chromatograph fitted with a 150 x 4.6 m.m. Ultrasphere - ODS column (Beckman Instruments, Inc., Irvine, CA). Elution was initiated with 85% methanol. After 0.5 minutes, the methanol concentration was increased to 100% over a period of 1.5 minutes. The flow rate was maintained at 1 ml/minute. Fractions (0.2 ml) were collected directly into minivials and 2 ml of Instagel scintillation cocktail (Packard Instruments

Co., Downers Grove, IL) was added. The radioactivity was assayed in a Beckman LS-9000 liquid scintillation counter at a tritium counting efficiency of 50%. Authentic BP metabolite standards were detected by UV absorbance at 254 nm.

RESULTS

The distribution of [G-^{3}H]BP was determined in the extracellular medium, nuclear, and cytoplasmic fractions of low passage and high passage cells, at specific time intervals following initiation of treatment. Cells were treated with 0.105 μM [G-^{3}H]BP for 12 hours, after which time the experimental medium was replaced with growth medium containing 13 μM unlabelled BP. Samples were removed at 24,48,72 and 96 hours following the initiation of [G-^{3}H]BP treatment.

Influx of [G-^{3}H]BP into the cytoplasmic fraction of HNF cells, 24 hours after carcinogen treatment was followed subsequently by an opti um appearance of the parent radiolabeled BP in the nuclear fraction at 48 hours (Table 1). Between 24 and 48 hours a large efflux of bound [G-^{3}H]BP from the cell into the extracellular experimental medium was observed. This release of radiolabeled BP then decreased as a function of time from 48 to 96 hours.

The nuclear, cytoplasmic, and extracellular medium fractions from treated cells were partitioned with ethyl acetate and the organic and water-soluble fractions separated. A major portion of [G-^{3}H]BP (>89%) was associated with the organic phase, and water-soluble metabolites constituted a minor portion (<11%) of radioactivity in all the fractions of both low passage and high passage cells (Table 1).

The non-covalently bound, ethyl acetate extractable PNH and it's metabolites were separated by HPLC, and the BP tetrols, diols, phenols, quinones and unmetabolized BP were identified by co-chromatography with authentic reference standards. The HPLC profile of [G-^{3}H]BP metabolites from the nuclear fraction of low passage cells (Figure 1), 72 hours after [G-^{3}H]BP treatment contained significant amounts of radiolabeled metabolites (>15%) and 78% of the counts were associated with the parent BP. Similar profiles were observed at the 24,48 and 96 hours time points, although

TABLE 1

Distribution of BP in Low Passage (LP) and High Passage (HP) Cells

	pmoles BP/10^6 Cells[a]							
	24 Hours		48 Hours		72 Hours		96 Hours	
	LP	HP	LP	HP	LP	HP	LP	HP
Nucleus								
organic soluble	1.96(94)[b]	2.24(94)	3.96(95)	2.79(98)	2.40(96)	1.60(94)	1.28(96)	1.44(99)
water soluble	0.13	0.14	0.23	0.05	0.10	0.10	0.05	0.01
Cytoplasm								
organic soluble	0.25(93)	0.42(91)	0.18(90)	0.24(96)	0.11(92)	0.23(89)	0.11(92)	0.17(90)
water soluble	0.02	0.04	0.02	0.01	0.01	0.03	0.01	0.02
Extracellular medium								
organic soluble	101.65(96)	114.58(98)	74.84(98)	118.91(96)	87.59(96)	94.77(97)	78.89(95)	81.45(91)
water soluble	4.47	2.90	1.72	5.23	3.42	2.56	3.79	7.66

a) NNF cells were incubated with [G-^{3}H]BP and the nuclear, cytoplasmic and extracellular medium fractions were prepared and extracted with ethyl acetate as described in "Materials and Methods". pmoles in each fraction were calculated by direct determination of radioactivity and data normalized to 10^6 cells.

b) The number in parenthesis correspond to percentage of counts in the organic solvent soluble fractions.

the percentage of metabolites obtained were less (Table 2). The nuclear fractions isolated from high passage cells had radioactivity which chromatographed mainly with the BP-phenol peak at all the time points and 77 to 88% of the counts eluted with the parent BP.

The nuclear fractions from low passage cells had approximately 4 times more radioactivity chromatographing with BP tetrols and approximately 4 to 12 times more radioactivity chromatographing with BP diols, compared to high passage cells (Table 2), at 72 and 96 hours after initiation of treatment.

TABLE 2

Distribution of BP and Metabolites in the Nucleus

	Percentage of Total Radioactivity							
	24 Hours		48 Hours		72 Hours		96 Hours	
	LP	HP	LP	HP	LP	HP	LP	HP
Tetrol	1.2	1.2	0.6	1.0	3.0	0.8	2.4	0.6
Diol	0.9	0.7	0.6	0.8	4.8	0.4	3.2	0.9
Unknown	1.1	1.3	0.7	0.7	2.0	2.0	2.4	1.5
Phenol	2.2	2.8	1.9	2.5	4.7	1.9	3.1	2.0
Quinone	1.1	1.4	0.6	1.5	0.6	1.0	0.85	0.6
BP	79.0	77.0	88.9	83.0	78.0	87.0	78.4	88.0

a) An aliquot of the ethyl acetate extract of the nuclear fraction was analyzed by HPLC and the percentage of total radioactivity recovered from the column which eluted with unmetabolized BP and with each of the standard metabolite peaks was determined.

b) BP-4,5 quinone, BP-7,8 quinone and 6-hydroxymethyl BP coelute at this position.

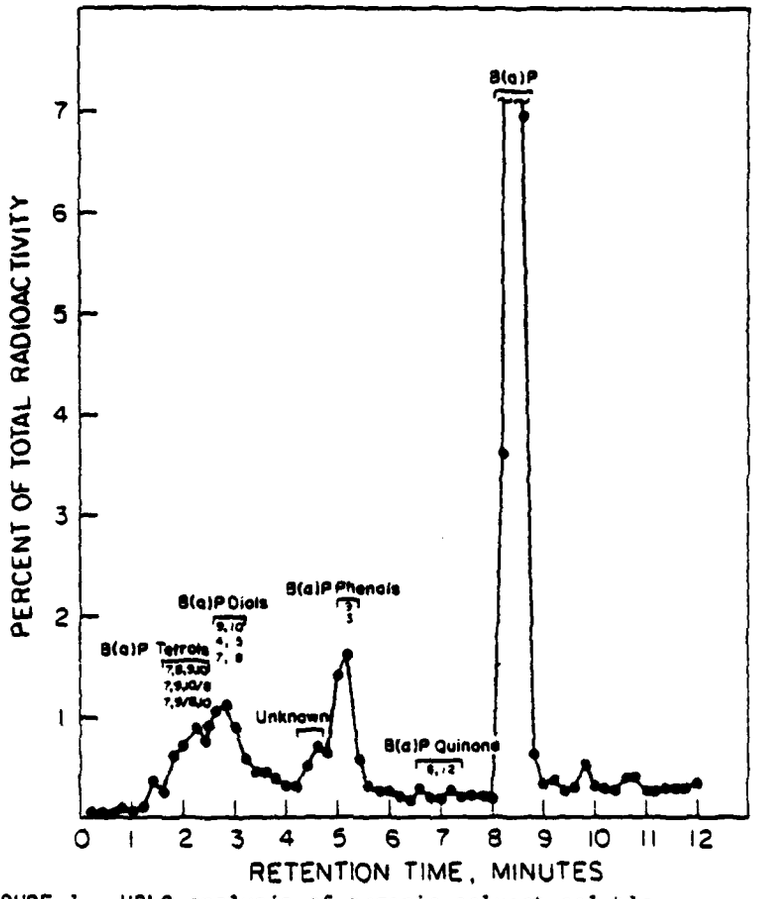

FIGURE 1. HPLC analysis of organic solvent soluble metabolites of [G-^{3}H]BP found in the nuclear fraction of HNF cells, 72 hours following initiation of treatment.

In the HPLC metabolite profiles of the cytoplasmic preparations the distribution of oxygenated metabolites was altered compared to the nuclear distribution over the 96 hour time period. There was a significant increase in the production of quinones specifically in the low passage cell populations, at the 24 hour time point. Moreover, the cytoplasmic fractions prepared from high passage cells exhibited a two fold increase in the radiolabel associated with BP-tetrols and BP-phenols compared to the low passage cells, at 48 hours and 96 hours after cell treatment (Table 3).

TABLE 3

Distribution of BP and Metabolites in the Cytoplasm

	Percentage of Total Radioactivity*							
	24 Hours		48 Hours		72 Hours		96 Hours	
	LP	HP	LP	HP	LP	HP	LP	HP
Tetrol	1.9	1.4	1.2	2.8	1.8	1.7	1.3	2.6
Diol	1.8	1.2	2.5	2.5	2.9	1.1	2.1	1.3
Unknown*	0.6	1.4	0.6	3.0	0.8	2.0	1.8	2.9
Phenol	1.2	2.0	1.4	3.3	2.0	2.7	1.3	3.1
Quinone	4.0	1.6	3.2	2.8	2.9	1.5	1.8	3.0
BP	82.9	80.7	84.7	66.3	83.0	75.6	86.5	70.0

* See legends a and b under Table 2

TABLE 4

Distribution of BP and Metabolites in the Extracellular Medium

	Percentage of Total Radioactivity*							
	24 Hours		48 Hours		72 Hours		96 Hours	
	LP	HP	LP	HP	LP	HP	LP	HP
Tetrol	0.5	0.4	0.4	0.9	0.7	1.0	1.2	2.1
Diol	0.5	0.5	0.4	0.9	0.6	0.8	0.7	1.2
Unknown*	1.6	1.2	1.4	1.4	1.1	1.1	1.0	1.6
Phenol	1.2	1.0	0.9	1.0	1.2	0.8	0.9	0.8
Quinone	0.4	0.3	0.3	0.3	0.5	0.2	0.2	0.4
BP	92.6	94.6	93.6	93.4	93.6	93.8	94.6	91.9

*See legends a and b under Table 2

There were no significant amounts of metabolites found in the extracellular medium of low passage or high passage cell cultures and greater than 90% of the radiolabel was associated with [G-^{3}H]BP (Table 4).

DISCUSSION

In this study, we have investigated the uptake and metabolism of BP in transformable (<PDL 6) and non-transformable (>PDL 20) human foreskin fibroblasts, 24 hours to 96 hours after carcinogen treatment. Cells exposed to a low concentration of [G-^{3}H]BP (0.105 µM) for 12 hours were subsequently treated with a high concentration (13 µM) of unlabelled BP for up to 96 hours to avoid a passive release of the [G-^{3}H]BP into the extracellular medium. The optimum uptake of [G-^{3}H]BP into the cytoplasm and localization into the nuclear fractions was observed at 24 hours and 48 hours after initiation of treatment. In our previous studies (12), the cells were continuously treated with a high concentration of [G-^{3}H]BP (13 µM). The optimum uptake of the PNH in the cytoplasm and nuclear fractions of these cell populations was observed at 12 hours and 24 hours following treatment.

When ethyl acetate extractable metabolites from the low passage and high passage cells were analyzed by HPLC, a major portion of the radiolabel in the extracellular medium (>90%), cytoplasm (>70%), and nucleus (>75%) chromatographed with the parent BP, at all the time points. A minor portion of the organic soluble counts in the cytoplasm and nucleus of the low passage and high passage cells chromatographed with the BP tetrol, diol, phenol, and quinone peaks.

There was a greater uptake of [G-^{3}H]BP by the nuclei of transformable cells, while efflux of the PNH into the extracellular medium of nontransformable cells was higher. Specifically, the uptake of [G-^{3}H]BP by the nuclei from LP cells was 1.5 fold greater than uptake by the HP cells. Moreover, 2 to 3 times more unmetabolized [G-^{3}H]BP was associated with the cytoplasm and extracellular medium of HP cells. These differences, although minor, may play a significant role in the ability of the low passage human skin fibroblasts to be transformed *in vitro*.

We have previously reported (12), that uptake of [G-^{3}H]BP by low passage HNF cells in culture is followed by binding to a cytoplasmic protein complex and transport to the nucleus within 24 hours. A major portion of the PNH (90%) was found bound to the cytoplasmic protein complex and eventually transported to the nucleus as unmetabolized BP (12). In addition, we found [G-^{3}H]BP bound to an acid extractable, non-histone, nuclear protein in the low passage cells, 24 hours after carcinogen treatment (J. Oldham, R. Tejwani and G.E. Milo, unpublished data). In the present study, 72 hours after initiation of [G-^{3}H]BP treatment, we observed an increase in the level of BP-diols and tetrols in the nuclear fraction of low passage cells. At this time, there was a decrease in the release of [G-^{3}H]BP into the extracellular medium of these cells. These data indicated a low level of metabolism taking place in the nucleus of the low passage cells, possibly at the chromatin level.

There was no significant difference in the cytoplasmic and medium metabolites produced by low and high passage cells at any of the time points studied. This further suggested little role for the microsomal AHH activity in formation of active metabolites of [G-^{3}H]BP in these cells. Also, when the water soluble metabolites, prepared by the procedure of Autrup (2) were analyzed by HPLC, no significant amount of metabolites were observed and 90% of the radioactivity eluted with the parent BP.

Activation of BP to a diol-epoxide is generally considered to be required for DNA binding leading to carcinogenesis (1). Since the amount of tetrols and diols in the transformable human skin fibroblasts is very low, formation of the diol-epoxide may not be necessary for macromolecular binding and subsequent carcinogenesis in these cells. Rogan and co-workers have observed horseradish peroxidase/H_2O_2 catalyzed covalent binding of BP to calf thymus DNA, and postulate that it occurs by one electron oxidation with the formation of a radical cation intermediate (10). In view of the strong electron donating properties of polycyclic hydrocarbons and the existence *in vivo* of several oxidants in the form of metal ion containing enzymes, one electron oxidation of the hydrocarbon to reactive radical cation intermediates capable of binding to cellular nucleophiles may constitute the critical first step in BP carcinogenesis in the low passage cells.

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HUMAN CELL NEOPLASTIC TRANSFORMATION WITH BENZO[A]PYRENE AND A BAY REGION REDUCED ANALOGUE OF 7,12-DIMETHYLBENZ[A]-ANTHRACENE

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INTRODUCTION

Polynuclear aromatic hydrocarbons (PAH) like benzo[a]pyrene (BP) and 7,12-dimethylbenz[a]anthracene (DMBA), have been shown to exert their toxic, mutagenic and carcinogenic activities after metabolic conversion to reactive intermediates (1,2,5,6,17,21). Our previous studies with BP have indicated that this carcinogen can induce neoplastic transformation in proliferating human skin fibroblast cells, when added during the S phase of the cell cycle (4,10). Although DMBA can induce a carcinogenic event in rodent cells in culture (12), this PAH does not induce such an event in proliferating human skin fibroblast cells in culture (4).

Previous reports from our laboratory have indicated that BP is taken up and initially bound to a cytoplasmic lipoprotein complex before being transported to the nucleus of human skin fibroblast cells in culture (4,19). DMBA, on the other hand, is randomly dispersed throughout these cells and is not bound to the cytoplasmic lipoprotein complex (4). These differences may in part explain the induction of neoplastic transformation in the normal human skin fibroblast cells by BP, and not by DMBA.

The bay region diol epoxide of DMBA, the 3,4-dihydrodiol-1,2-epoxide, has been proposed to be the major metabolite responsible for the mutagenic and carcinogenic activities of this PAH (3,18). Consistent with this proposal, DMBA exhibits mutagenicity in the Ames assay only in the presence of a microsomal activation system (7).

NEOPLASTIC TRANSFORMATION

The observation that 1,2,3,4-tetrahydro-DMBA (TH-DMBA) was mutagenic in the absence or presence of a microsomal activation system, using three strains (TA1537, TA98, TA100) of S. typhimurium (7), provided impetus to study this A-ring reduced analogue using human cells. Since TH-DMBA does not yield a bay region diol-epoxide unless it is first oxidized (aromatized) to DMBA, it can serve as a useful probe for mechanistic investigations of the transformation phenomena.

In our laboratory, we have defined several indices of human fibroblast cell transformation in response to a variety of chemical carcinogens. These include morphological changes, extended life span, growth in culture conditions toxic for untreated normal cells, increase in lectin agglutinability and alteration in the cellular prostaglandin levels. The anchorage-independent growth of transformed cells in soft agar has been the most consistent and reliable indicator of tumor production in athymic nude mice and on the chick embryonic skin organ culture system.

In this report, data are presented on the carcinogenicity of TH-DMBA in human neonatal foreskin (HNF) fibroblast cells in culture.

MATERIALS AND METHODS

Chemicals

DMBA was purchased from Eastman Chemical Company and BP was obtained from the National Cancer Institute Repository. TH-DMBA was synthesized by a method previously described (20) and was checked for purity by HPLC prior to use. The hydrocarbon was purified on a Spherisorb ODS 5μ column (4.5 mm x 25 cm) using a linear gradient of 25-100% methanol for 1 hour. All solvents were of reagent or analytical grade.

Cell Cultures and Treatment of Cells with PAH

Primary cultures of human HNF cells were established as described previously (15). Proliferating HNF cell monolayers between population doubling (PDL) 4 to 5 were blocked in the G_1 phase of the cell cycle by feeding the cultures with a non-proliferating Dulbecco's modified Eagle's medium deficient in arginine and glutamine (8,10). After twenty four hours, when the mitotic index was 0.1% to 0%, the non-proliferating medium was removed and the cultures were

treated with 3.2 µg/ml of BP, 1µg/ml of DMBA or 0.5 µg/ml of TH-DMBA in acetone. Cell cultures to which an equal volume of acetone was added served as controls. The radiolabeling index of the cells pulsed with ^{3}H-thymidine at this time was 90% (10). The carcinogen was allowed to remain in contact with the cells during the S phase of the cell cycle which was 8.2 hours long.

Selection of Transformed Cell Populations

The treated cell populations were serially passaged into complete growth medium supplemented with non-essential amino acids and vitamins for 16 PDL (8,10). The treated cell populations were then seeded into soft agar.

Anchorage Independent Growth

The treated cell populations were trypsinised and single cell suspensions, in 0.3% soft agar and Dubecco's LoCal complete growth medium, were seeded over a 2% agar base containing RPMI 1629 complete growth medium. The soft agar cultures were incubated at 37°C in a 4% CO_2 atmosphere (8,10). Three weeks later, large well defined boluses were trnasferred to a 75 sq. cm. flask and grown to a saturation density. The cells were serially passaged and maintained in the selection medium until they were seeded on the chick embryonic skins to evaluate neoplasia.

Neoplastic Transformation

10^5 cells from treated and control cultures at PDL 36 were seeded on the chick embryonic skin organ cultures (CES) prepared from 9 to 18 days old fertilized eggs (9). The CES were layered onto an agar base containing 10 parts of 1% Agar in Earle's balanced salt solution, 4 parts of four day old chick embryo extract and 4 parts of fetal bovine serum. The skins were removed and fixed in Bouin's solution. Five micron transverse sections were stained with hematoxylin and eosin and examined for the presence of invasive features.

RESULTS AND DISCUSSION

TH-DMBA was mutagenic in the Ames assay both with and without metabolic activation in three strains of S.

typhimurium (7). In fact, this A-ring reduced analogue was more mutagenic in the absence of microsomal activation using the plasmid deficient strain TA1537 (7). TA1538 and the missense tester strain TA1535, which are relatively insensitive to DMBA mutagenesis were not mutated by TH-DMBA (7). Nonetheless, DMBA required metabolic activation, by addition of the S9 microsomal fraction, in order to elicit mutagenicity in strains TA1537, TA98 and TA100 (7), whereas TH-DMBA required no such metabolic activation. TH-DMBA, in the absence of the S9 fraction had mutagenic properties similar to metabolically activated DMBA. This is a marked departure from other PAH which require an activating system or chemical modification to a reactive species to effect mutagenesis in the Ames assay. Thus, even 3,4-dihydrodiol-7-methylbenz(a)anthracene was nonmutagenic in the strain TA98, when cofactors required for the microsomal activation system were omitted (11).

Our previous studies have indicated that when HNF cells are treated with BP, a major portion of the PAH is bound to a cytoplasmic lipoprotein complex and is subsequently transported to the nucleus as the parent compound (4,19). HPLC analysis of the BP metabolites covalently bound to DNA has indicated a low level of BP metabolism taking place in these cells, with the formation of a small amount of the BP-7,8-diol-9,10-epoxide-I-deoxyguanosine adduct (19). In the present study, BP also induced neoplastic transformation in the HNF cells and the treated cell populations (dose 3.2 µg/ml) exhibited anchorage independent growth with a frequency of colony formation of $86/10^5$ cells seeded in soft agar (Table 1). These transformed cell populations were invasive on CES and produced a fibrosarcoma.

Although DMBA did not transform human foreskin fibroblasts in culture as measured by anchorage independent growth, cells transformed by TH-DMBA (dose 0.5 µg/ml) grew to spherical colonies 10 to 12 days after seeding on soft agar. Also, the frequency of colony formation of TH-DMBA treated cells was $84/10^5$ cells seeded on soft agar (Table 1). The TH-DMBA treated cell populations exhibiting anchorage independent growth were invasive on the CES and produced a fibrosarcoma.

Thus, in conclusion, DMBA does not transform HNF cells in culture as measured by anchorage independent growth. It is interesting to observe that TH-DMBA, the analogue of DMBA completely reduced in the bay region, can induce neoplastic transformation in these cells and is as potent as BP, at

TABLE 1

Characterization of Transformed Human Foreskin Fibroblast Cells through Anchorage Independent Stage and Neoplastic Stage of Carcinogenesis.

Compound	(μg/ml)	Evidence of Colony Formation in Soft Agar	Incidence of Tumor Formation in CES
BP	3.2	86	1/1*
7,12 DMBA	1	0	N.D.
TH-DMBA	0.5	84	1/1

*BaP treated cells were also evaluated for tumor formation in nude mice (10).

1/6 the dose of BP used (Table 1). This pronounced activity of TH-DMBA is similar to what would be expected in our system if we were assessing an ultimate carcinogen. Investigations with radiolabeled TH-DMBA are under way to assess further whether metabolism and covalent binding to macromolecules in the nucleus are involved in the transformation event.

Previous reports have indicated that 1,2,3,4-tetrahydro-7-methylbenz[a]anthracene, a 12-desmethyl analogue of TH-DMBA, is non-carcinogenic (16). It is known that the 12-methyl function in DMBA provides sufficient steric interaction with the C_1-carbon-hydrogen of the A ring to stabilize a ketone function at position 5 owing to decreased planarity of the tetracyclic system (13,14). In TH-DMBA the C_{12}-C_1 interaction freezes the ring A in a half-chair conformation with C_2 slightly and C_3 markedly out of plane with the aromatic anthracene system. The speculative A-ring triol of DMBA, possibly arising by reaction of DNA or other macromolecular nucleophile with the proposed bay-region 3,4-dihydrodiol-1,2-epoxide, is expected to have a similar conformation.

These observations question the necessity for metabolic activation in the case of TH-DMBA as a prerequisite to either macromolecular binding or transformation in human cells, but further work employing radiolabeled TH-DMBA is required before this possibility can be adequately assessed.

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CHEMICAL PROPERTIES OF BACTERIAL MUTAGENS IN STACK COLLECTED COAL FLY ASH

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INTRODUCTION

Previous studies have shown a size classified, stack collected sample of coal fly ash to contain bacterial mutagens (1,5,6). The mutagenic material is composed largely of direct acting frameshift mutagens, as shown by activity in the Ames test using Salmonella typhimurium strains TA 98 and TA 1538 without metabolic activation by S9 and a lack of activity in strains TA 1537 and TA 1535 with or without S9 (1). A slight increase in the activity of the mutagenic material is obtained by addition of S9 to the Ames test cultures (5). The presence of polynuclear aromatic hydrocarbons (PAH) is expected in coal fly ash and these compounds have been tentatively identified in the samples used in this study (6). Unsubstituted or alkyl PAH may account for the increase in activity on addition of S9, however the direct acting mutagens cannot be unsubstituted PAH or alkyl PAH since these require the presence of S9 for activation.

Considering that several million tons of coal fly ash are emitted annually to the atmosphere and transported over long distances, the chemical properties and identity of the mutagenic material in the ash are of great importance in estimating the effects of increased coal combustion on human health.

The mutagenic material in the coal fly ash samples studied in this work shows about twice the activity in serum as in cyclohexane extracts of the fly ash and about ten times more activity results from extraction into serum than into phosphate buffered saline (PBS) (1). In serum, most of the mutagens are bound in high molecular weight complexes with protein (1). The activity of the serum extract is increased by the addition of ethylenediamine tetraacetic acid (EDTA), an effect which has been suggested as being due to chelation of mutagenic metal ions from the serum proteins (1).

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CHARACTERIZATION OF HUMAN CELLS TRANSFORMED BY CHEMICAL AND PHYSICAL CARCINOGENS IN VITRO

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SUMMARY

Several different classes of chemical carcinogens induced the transformation of human fibroblasts grown in vitro. Characteristics of the events that occur from time of treatment through the expression of neoplastic transformation are presented. The S-phase appeared to be the portion of the cell cycle most vulnerable to insult. Staging of the cells by blocking them in G_1 before releasing them to proceed through scheduled DNA synthesis (S) was required to induce reproducible transformation. Compounds such as insulin were added to the cells upon release from the block to sensitize the cells to the carcinogen that was added during S. Growth of the transformed cells as distinct from nontransformed cells was promoted by growth in medium supplemented with 8X nonessential amino acids. Carcinogen-treated cells in the early stage of transformation exhibited abnormal colony morphology and were able to grow at 41° C, in air atmosphere, and in medium supplemented with only 1% serum. In addition, the transformed cells were insensitive to KB cell lysate and exhibited density independent, as well as anchorage independent, growth (i.e., growth in 0.33% agar). Cells that grew in soft agar also produced undifferentiated mesenchymal tumors in preirradiated nude mice.

Key words: human cells; chemical carcinogen; transformation; in vitro.

INTRODUCTION

We have reported previously the neoplastic transformation of human diploid cells in vitro with a number of different carcinogenic agents including chemical carcinogens (1), transforming virus (2), and physical carcinogens such as ultraviolet light and ^{137}Cs gamma irradiation (3) (^{137}Cs = 662 keV monoenergetic source at 56 rad/min). A number of compounds have been used to sensitize the population of susceptible cells that were in S when exposed to the carcinogen insult (4). However, these "sensitizing" compounds by themselves are not carcinogenic. We have examined the events that occur after the chemical insult that initiates the expression of neoplastic transformation, and the data presented here will identify the variations that occur with time in culture as the transformed cells express their neoplastic character.

MATERIALS AND METHODS

Cell culture. Neonatal foreskin (NFS) cell cultures were established as described previously (5,6). Fibroblasts were separated immediately from the mixed cell culture by selective detachment from the substratum. The fibroblast cultures were passaged routinely using 0.1% trypsin and were maintained on Eagle's minimum essential medium (MEM) prepared with Hanks' balanced salt solution, 25.0 m*M* HEPES buffer

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(GIBCO, Grand Island, NY) at pH 7.2, supplemented with 0.1 m*M* sodium pyruvate, 1X nonessential amino acids (Microbiological Associates, Rockville, MD), 2.0 m*M* glutamine (Micro. Assoc.), 0.2% sodium bicarbonate, 5.0 μg/ml gentamicin, and 10% fetal bovine serum (FBS, Rehsis, Kankakee, IL). This growth medium was designated CM. The cultures were incubated in an atmosphere of 4% CO_2-enriched air at 37° C.

Cytotoxicity assays. To determine the transforming dose to be used in the experiments it was necessary to examine the cytotoxic effects of the carcinogens on the cells at risk. Cells were seeded at 40/cm² in MEM supplemented with 1X essential amino acids (Micro. Assoc.), 1X vitamins (Micro. Assoc.) and 20% FBS; this medium was designated CMV. After attachment the cells were treated for 24 h with varying doses of a carcinogen (all carcinogens furnished by IIT Research Institute Chemical Repository, Chicago, IL) in CM containing 10% FBS. The treatment medium was removed and the cells were washed to remove the carcinogen. The cultures were refed with CMV and incubated for 7 to 13 d. Colonies were fixed with 3% buffered formalin and stained with hematoxylin; those with ≥100 cells were scored to obtain the relative colony forming efficiency (RCE, the number of treated colonies relative to the number of untreated colonies). The dose (ED_{10}), which yielded a 50% reduction from control values in the RCE, was then calculated.

In several experiments, immediately after carcinogen treatment (ED_{10}, ED_{25}, ED_{50}) as described below in the protocol for transformation, the cells were removed by trypsinization and plated at 40/cm² in CMV. The cultures were then allowed to proliferate for 7 to 13 d and the RCE determined as just described. This procedure also permits selective removal of the abnormal colonies for continued passaging.

Transformation protocol (Fig. 1). Neonatal foreskin fibroblasts at a population doubling (PDL) of ≤5 were passaged at 5000 cells/cm² into MEM minus arginine and glutamine (Biolabs, Northbrook, IL), supplemented with 1.0 m*M* sodium pyruvate, 5.0 μg/ml gentamicin, 0.2%

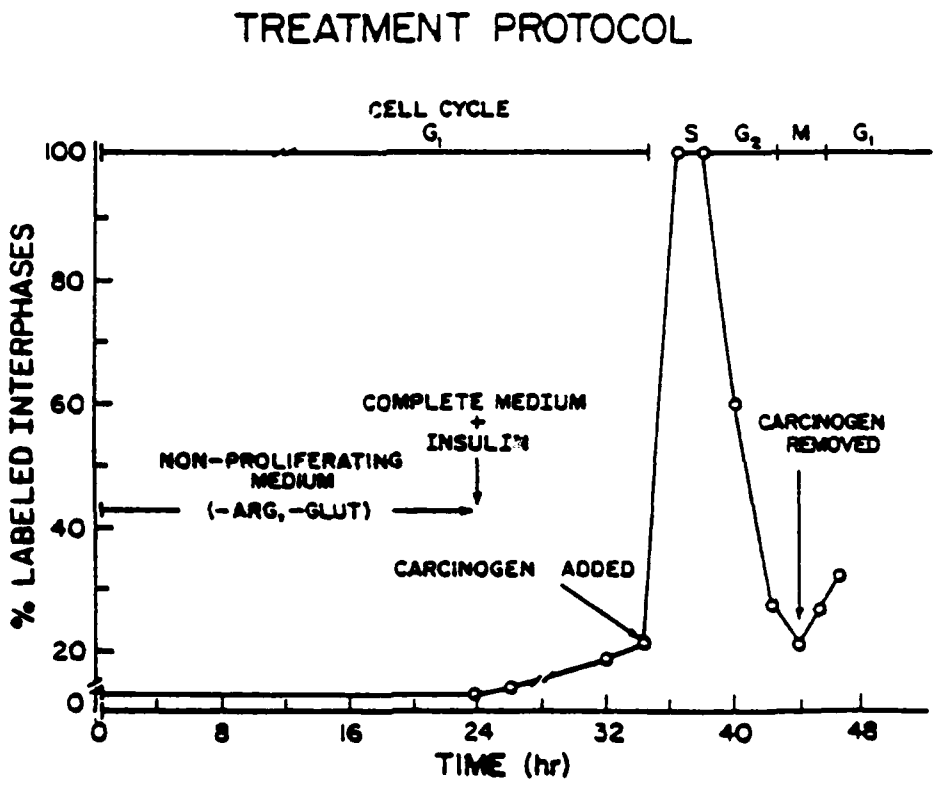

FIG. 1. Treatment protocol for the transformation of human fibroblasts in vitro. The percent labeled interphases is plotted as a function of time during synchronization and carcinogen treatment.

sodium bicarbonate, and 10% dialyzed FBS. This deficient blocking medium was designated DM. Immediately before the cells were prepared for seeding, the confluent cultures were washed with DM minus serum. Cultures that had been confluent for >24 h were not used. Trypsin (0.1%) used in these experiments was prepared in DM minus serum. The cells were recovered by centrifugation at 600 ×g and the cells were seeded immediately in DM. After 24 h incubation in a 4% CO_2 environment, the cells were released from the nonproliferating state by changing to CM containing 10% FBS and 0.5 U/ml insulin (IN). Data concerning potentiation of transformation by other compounds such as 17-β-estradiol, phorbol myristate acetate, anthralin, et cetera, are presented elsewhere (4). With this protocol, the cells became synchronous (1). Cells in early S were treated with the carcinogen at a biological toxic dose (ED_{50}). Ten hours after removal of the block, with 20% of the cells in S, carcinogens with a long half-life were added to the cell cultures. Carcinogens with a short half-life were added 2 h later when the major portion (>90%) were in S. The cells were exposed to the carcinogens for time periods ranging from a few minutes for carcinogens with a brief half life, up to 12 h for carcinogens with a long half-life.

Treatment was terminated when the experimental agent was removed, and the cells were passaged at a 1:2 split ratio into CM containing 2X vitamins, 8X nonessential amino acids, and 20% FBS, hereafter referred to as 8X medium. When these and subsequent cultures reached confluent density (80 to 90%), they were passaged at a 1:10 split ratio into 8X medium. Cultures were never allowed to remain at confluency for more than 24 h.

Characterization of the Transformed Cells, Early Stage (Fig. 2)

Lectin agglutination. Carcinogen-treated (5 to 7 PDL after treatment) and control fibroblast populations were collected by trypsinization and washed three times with Dulbecco's phosphate buffered saline (PBS). The cell density was adjusted to 10^6 cells/ml in PBS, and 0.18 ml of the suspension was added to microtiter wells. Wheat germ agglutinin (0.04 ml) was then added at serial concentrations from zero to 2500 μg/ml (final volume in each well of 0.22 ml). Positive reactions were visible under the microscope within 10 min (4,7).

Altered colony formation. Four population doublings after carcinogen treatment, the cells were seeded into 60-mm d wells at 1000 cells per well in 8X medium. After incubation for 7 to 13 d, the cells were fixed with 3% PBS buffered formalin, stained with hematoxylin, and examined for abnormal colony morphology. Abnormal colonies exhibited loss of parallel orientation, cross-cross piling up of the cells, and loss of the whorling pattern of alignment that normal fibroblasts exhibit in a density dependent state.

Growth at 41° C, in 1% serum, or in air atmosphere. Carcinogen-treated (four PDL after treatment) and control fibroblast cultures were passaged at 5000 cells/cm^2 under one of three conditions: (a) at 41° C in CM, 4% CO_2 (3); (b) in CM, but with 1% FBS, at 37° C, 4% CO_2 (8); or (c) in air atmosphere (not CO_2 enriched) in CM and at 37° C. The cultures were passaged routinely at 1:4 split ratios when confluent, and they were maintained under the altered growth conditions to observe any differential growth

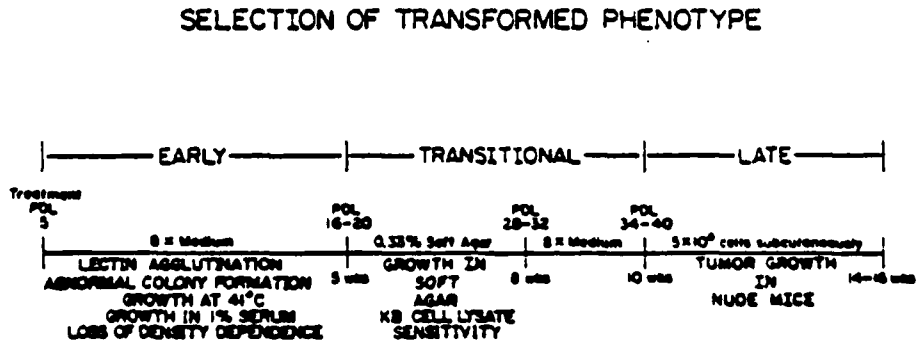

FIG. 2. Characterization and selection of transformed human fibroblasts as transitioned from the early to late stages of carcinogenesis.

characteristics between the treated and control cell populations.

Characterization of the Transformed Cells, Transitional Stage

Growth in soft agar. Carcinogen-treated and control fibroblast populations were passaged in 8X medium at 1:10 split ratios until they reached PDL 20 to 24. They were then seeded at 50,000 cells/25 cm^2 area in 2 ml 0.33% agar prepared in Dulbecco's LoCal medium (Biolabs) supplemented with 1 m*M* sodium pyruvate, 1X nonessential amino acids, 1X essential amino acids, 2.0 m*M* glutamine, 1X vitamins, 0.2% sodium bicarbonate, 5.0 μg/ml gentamicin, and 20% FBS. These cells were layered over 5 ml of a 2% agar base prepared in RPMI 1629 medium (GIBCO) supplemented with 1X sodium pyruvate, 1X nonessential amino acids, 1X essential amino acids, 2.0 m*M* glutamine, 1X vitamins, 0.2% sodium bicarbonate, 5 μg/ml gentamicin, and 20% FBS. The cultures were kept in a high humidity incubator in a 4% CO_2 atmosphere; 0.5 ml of 0.33% agar (prepared in supplemented LoCal medium) was added every 3 to 5 d. The cultures were examined for colony (bolus) growth weekly, and they were considered negative when no growth was seen in 4 wk. Cultures were scored as positive when colonies of ≥50 cells were observed and the number of colonies per well were counted. Monolayer cultures were then reestablished by removing the colony from the agar with a tuberculin syringe (20 gauge needle) and seeding it into 8X medium. After attachment and sufficient growth, the cells were removed by trypsinization and reseeded to distribute evenly the cells from the monolayer colonies that had developed.

Cloning of transformed cells in medium containing heparin and KB cell lysate. Sterile heparin (Upjohn Company, Kalamazoo, MI) was diluted to 1 mg/ml in culture medium. KB cells were grown to confluent monolayers in MEM supplemented with 15% FBS. Twelve to twenty-four hours after reaching confluence, cells were collected, resuspended to 10^7 cells/ml in supernatant medium and stored at −70° C. Approximately 2 to 6 h prior to use, KB cells were frozen-thawed twice, and diluted to give a lysate from 2×10^6 total cells in a volume of 0.2 ml.

Chemically treated cells that were passaged through soft agar were plated at 2000 cells/60 mm dish in 3 ml MEM supplemented with 15% FBS and 0.15% $NaHCO_3$. After 6 h, heparin was added at the indicated concentrations in an additional 3 ml of medium. After 7 d, these cultures were refed with medium still containing the heparin. Other cultures were treated with a 0.2 ml volume of diluted KB cell lysate. Lysate and medium were removed after 3 d of treatment and the cells were refed with culture medium only. All dishes were fixed and stained 12 to 14 d after initial plating.

Characterization of the Transformed Cells, Late Stage

Tumor growth in nude mice. Nude mice (Sprague-Dawley, Madison, WI), five times backcrossed, were obtained at 6 wk of age. Between 10 and 12 wk of age, the mice were irradiated (whole body) with 450 rad (from a ^{137}Cs source) 48 h prior to the subcutaneous injection of $5X\ 10^6$ carcinogen-treated or control cells suspended in CM medium. (Other procedures that have proved useful for the inoculation of transformed cells are: (a) to suspend 5×10^6 cells in 0.5 ml of 0.33% agar supplemented with CM, and (b) remove the large colonies from the soft agar, centrifuge at 250 ×*g*, resuspend in CM, and inoculate subcutaneously into the subcapsular region of the mouse.) Preliminary inocula of 5×10^7 down to 10^6 cells per injection indicated that 5×10^6 cells was a sufficient inoculum size. The treated cells for injection were those reestablished in monolayer culture from colonies removed from soft agar. Control cells were of similar PDL. Blebs created by the injection usually regressed in 24 to 48 h, and developing tumors were evident 2 to 4 wk after injection. No tumor development by 6 wk was considered a negative result. Tumors were excised from the animals and sent to the pathology laboratory for histological evaluation, or the cells were again established in culture (2,4).

Tumor cells were recultured by excising the tumor, mincing it into small pieces (1 to 2 mm^2) and incubating the tissue with 0.25% collagenase (in CMV) for 2 h. The cells were then removed from the collagenase-containing medium by centrifugation (600 ×*g*, 7 min) and seeded into CMV to which 4% "antimouse" fibroblast serum had been added. This serum was prepared by five injections, 1/wk for 5 wk, of skin fibroblasts from nude mice into the footpads of white New Zealand rabbits. One week after the last injection, the rabbits were bled, and the "antimouse" serum was prepared. Homogeneous populations of mouse

roblasts were killed within 3 to 5 d when grown in the presence of this serum, but human fibroblast populations were not affected. Therefore, primary cultures of cells derived from the tumors were grown in the presence of the "antimouse" serum for 7 d to eliminate mouse fibroblast contamination of the cultures. The tumor cells recovered from the mouse were used to determine the karyology of the cells (2).

RESULTS

Early stage. Toxicity values were obtained (6), identified as effective inhibitory dosages (ED), and used to treat cell populations at high cell densities (5000 cells/cm²) as described in the transformation protocol. In all experiments where toxic values of the carcinogen were used that exceeded the ED_{50} values, without exception, the incidence of transformation as measured by the presence or frequency, or both, of formation of abnormal foci or altered lectin agglutination profiles, or both, dropped rapidly to zero (control values). To evaluate the toxic effect of the carcinogens on cells at either low cell density or high cell density the cells were treated in the following manner: Cells treated in S at 5000 cells/cm² were cultured at 40 lls/cm² immediately after discontinuation of the rcinogen treatment. Cells treated at low cell density (nonsynchronized) were fed continually for 9 to 11 d once the carcinogen treatment was discontinued. The profiles of survival were similar in each treatment situation. We did observe that the cells treated in S at a high cell density and subsequently grown at a low cell density were more sensitive to the toxic effect of aflatoxin B_1 (Fig. 3). This type of toxic response (more susceptible to the effects of the carcinogen during S) was also observed for the other carcinogens (Table 1), including UV.

Cell populations treated with either beta-propiolactone (β-PL), aflatoxin B_1 ($AF\text{-}B_1$), propane sulfone (PrS), 4-nitroquinoline-1-oxide (4-NQO), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), ethylmethane sulfonate (EMS), benzo(a)pyrene [B(a)P], or UV light at 254 nm were able to grow at 41° C after five PDL, whereas normal untreated cultures would not. The treated cell populations were serially passaged at 41° C for 4 or 5 passages; they were then returned to 30° C and grew to an extended life span of 100 to 120 PDL. The transformed cells were able to grow in 1% FBS-supplemented CM at a rate equivalent to normal cells growing in

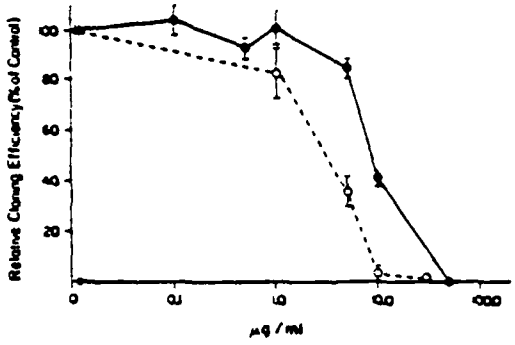

FIG. 3. Cytotoxicity of *aflatoxin-B*, on human fibroblasts. ●—●—●, Cells seeded at 40/cm² (nonsynchronized low cell density were treated with different concentrations of $Af\text{-}B_1$. After the removal of the carcinogen, the cells were refed with CM for 9 to 11 d. The colonies were fixed, stained, and enumerated to obtain the relative cloning efficiency as the percent of control. O—O—O, Cells were synchronized at a high cell density (5000/cm²), released from synchrony, and treated during S with different concentrations of $Af\text{-}B_1$. After removal of the carcinogen, the cells were harvested by trypsinization, reseeded at 40/cm² and refed with CM for 9 to 11 d before determining the relative cloning efficiency.

10% FBS-supplemented CM. Normal cells that were passaged 1:4 in 10% FBS supplemented CM took 4 d to reach a confluent density. Transformed cells split 1:10 in 1% FBS supplemented CM reached a confluent density in 5 d (1). Cloning of treated cells at a density of 1000 cells/cm² revealed the presence of abnormal colonies (Fig. 4). It presumably was from these colonies that subsequent cell populations gave rise to cells that exhibited altered lectin agglutination profiles and were able to grow in soft agar and produce tumors in nude mice.

Transformed cell populations have been shown to exhibit altered lectin agglutination profiles during the early stage of transformation (2). A comparison of the values for lectin agglutination, growth in soft agar, and tumor formation, all presented in Table 1, suggested that differential agglutination profiles were characteristic for the early stage of the carcinogenic process. An exception was with *N*-acetoxy-2-acetylaminofluorene (*N*-Ac-AAF) where treated cells exhibited altered lectin agglutination (39 μg/ml) in the early stages of the process, but in four out of five attempts these cells would not passage through soft agar. In all of the transformed cell populations examined for altered agglutination profiles, the amount of wheat germ lectin needed to agglutinate the cells was reduced from 2500 μg/ml

TABLE 1

CHARACTERISTICS OF TRANSFORMED HUMAN SKIN FIBROBLASTS DURING TRANSITION FROM THE EARLY PERIODS OF THE TRANSFORMATION PROCESS THROUGH TO NEOPLASIA

Carcinogen	ED_{50}[a]	LA[b]	SA[c]	TI[d]
	μg/ml			
β-P1	13.0	ND[e]	14.0	3/4
AF-B_1	10.0	78	10.0	8/14
PrS	5.0	125	20.0	7/11
4-NQO	0.002	39	0.1	2/4
MNNG	0.5	39	1.0	3/5
EMS	10.0	ND	20.0	2/4
1-NA	65.0	19	1.5	2/9
2-NA	68.0	250	0.1	1/6
N-OH-1-NA[f]	2.0	ND	30.0	1/8
N-OH-2-NA[f]	1.7	ND	29.0	2/7
N-0-1-NA[g]	27.5	19	5.1	3/16
N-0-2-NA[h]	23.1	250	1.0	3/16
N-Ac-AAF	0.5	39	0	0/8
Hydrazine	35.0	19	9.2	4/8
UDMH	50.0	ND	5.1	4/8
MAMA	3.6	ND	900.0	2/16
BtaIP	10.0	39	1.0	6/10
MMS	0.1	2500	0	0/6
UV	40 j/m^{-2}	78	20.0	4/6
^{137}Cs	100 RAD	39	13.1	3/7
Control	—	2500	0	0/10

[a] Cells were seeded at 40/cm^2, treated for 24 h with varying doses of the carcinogen, and allowed to grow 7 to 13 d before scoring the colonies to determine the ED_{50}.

[b] These values in micrograms per milliliter of lectin, were obtained using wheat germ agglutinin and represent the lowest concentrations that will agglutinate the cells in 10 min. LA = lectin agglutination.

[c] Fifty thousand cells at PDL 20 were seeded into 0.33% agar (SA) supplemented with LoCal + 20% FBS, overlaid on a 2.0% agar base supplemented with RPMI 1629 + 20% FBS. The colonies were counted after 28 d. The incidence is expressed as the number of colonies per 10^6 cells.

[d] The tumor incidence (TI) is expressed as a fraction: the numerator is the number of mice giving rise to tumors 0.8 to 1.2 cm in size, 4 to 6 wk after the injection of 5×10^6 cells, and the denominator is the total number of preirradiated (450 whole body) mice injected with a given cell population.

[e] ND = Not determined.

[f] Cells were exposed to these compounds at pH 5.0.

[g] *N*-0-1-NA = *N*-phenyl-1-naphthylamine.

[h] *N*-0-2-NA = *N*-phenyl-2-naphthylamine.

to 19 to 250 μg. Another lectin that was found to differentiate between normal and transformed cells was castor bean lectin, but soybean or peanut lectin did not do so (unpublished data).

Transitional stage. Cell populations transformed with either AF-B_1, MNNG, PrS, or 4-NQO were serially passaged to either 5, 10, 15, or 20 PDL before seeding into soft agar to establish the point during the transformation process at which the cells acquired the property of growth in soft agar. The treated cells would not grow in nude mice at this stage of the transformation process. We found that the cells must be passaged (time in culture) through at least 16 and preferably 20 PDL before they would grow in soft agar. Exceptions were found among cells treated with the physical carcinogens, UV light and ^{137}Cs; these transformed cell populations appear to require 20 to 25 PDL in culture. Another interesting finding was that the *N*-hydroxylated naphthylamines were more effective in inducing cells to grow in soft agar when the cells were exposed to the compounds at pH 5.0; cells treated with this family of carcinogens at pH 5 and serially passaged to 20 PDL exhibited a 3- to 4.5-fold increase in colony formation in soft agar over cells exposed to the carcinogen at pH 7.0 (9).

A 0.33% agar supplemented with LoCal medium over a 2% agar base containing RPMI 1629 growth medium provided the optimum conditions for growth in soft agar (Table 2). The frequency of colony formation by AF-B_1-treated cells in a 1% methyl cellulose also was optimized in LoCal medium (23 colonies in

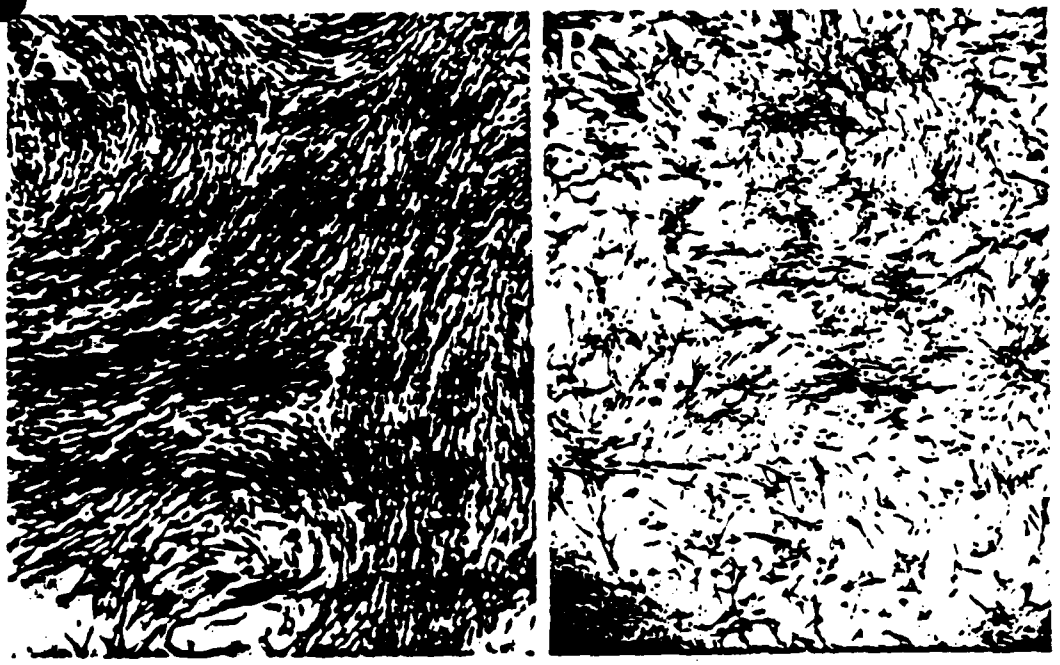

FIG. 4. Cells were seeded at four PDL at 1000 cells/25 cm² into 8X medium and allowed to grow into colonies (13 d). *A*, Colony morphology typical of that formed by normal fibroblasts. *B*, Colony morphology typical of that formed four PDL following treatment of the cells with Af-B, (10.0 μg/ml). ×25.

00,000 cells seeded; data not shown). However, there was no distinct advantage in using methyl cellulose as an anchorage-independent growth medium instead of soft agar.

Another technique used to characterize the transitional stage of the transformation process was to measure the response of transformed cells to varying concentrations of different low molecular weight carbohydrates. *N*-Ac-AAF, PrS, and Af-B, transformed cells that produced colonies in soft agar did not exhibit an altered toxic response when treated with either Con-A, DEAE-dextran, or dextran-sulfate (data not shown). However, when cells of equivalent passage were treated with 25 μg/ml of heparin, the treated cells were more resistant than the untreated controls (Table 3). We also observed a differential response to KB cell lysate. Untreated cells were more sensitive to lysate from 2×10^6 KB cells than the transformed cells. This is in agreement with a previous report that chemically transformed hamster cells are more resistant than normal cells to heparin (10). Also lysate from transformed hamster cell lines enhances the plating efficiency of other transformed hamster cell lines, but inhibits normal cell growth (unpublished data, G. G. Hatch).

Late stage. After growth in soft agar and reseeding into monolayer culture, 34 to 40 PDL after carcinogen treatment was discontinued, the cells were injected subcutaneously into the subscapular area of nude mice. These mice had been irradiated 2 d previously with 450 rad of ^{137}Cs. Tumor incidence varied among populations of carcinogen-treated cells. The centers of the nodules, 0.6 to 1.05 cm d (Fig. 5), were necrotic

TABLE 2

INCIDENCE OF GROWTH OF AFLATOXIN B, TRANSFORMED CELLS IN SOFT AGAR SUPPLEMENTED WITH DIFFERENT GROWTH MEDIA

Composition of Agar		
2% Base	0.33% Overlay	Frequency[a]
MEM	MEM	0.01
RPMI 1640	MEM	0.01
RPMI 1640	LoCal	0.1
RPMI 1629	RPMI 1640	1.0
RPMI 1629	LoCal	10.0

[a] Cells were seeded in 25 cm² wells at 50,000 cells/well. Frequency is expressed as the number of colonies formed per 10^6 cells. The cells were treated at PDL 5 with 10 μg/ml Af-B$_1$, and seeded into the soft agar at PDL 20.

TABLE 3

RESISTANCE OF NORMAL OR TRANSFORMED CELLS TO HEPARIN OR KB CELL LYSATE[a]

Treatment	Passage Level	Cloning Efficiency	Heparin			KB Lysate Cell Equivalents × 10^6				
			400	100	25	6	2.0	1.0	0.6	0.25
			$\mu g/ml$							
None	32	3	7[b]	15	27	101	32	68	85	96
AF-B_1	23	7	3	24	73	95	51	91	85	81
N-Ac-AAF	23	6	4	47	86	83	83	102	101	99
PrS	25	6	4	55	95	110	71	95	106	102
4-NQO	36	5	7	57	58	68	60	76	81	97
β-PL	46	8	19	84	98	100	85	102	105	95
1-NA	53	8	40	89	93	84	98	97	97	89
2-NA	60	9	24	108	127	130	112	121	93	115

[a] Human cell populations transformed by *N*-Ac-AAF, PrS, 4-NQO, β-PL, 1-NA, 2-NA, or Af-B, serially passaged to PDL 20. The concentrations of carcinogens used to transform the cell populations were reported in Table 1 (ED_{10} values). The transformed cells were passaged through soft agar (0.33%), repopulated and used during the transitional stage. The control cells (noncarcinogens treated) were serially passaged to comparable PDL.

[b] Percent survivors, based upon the cloning of untreated cells.

with dense infiltration of neutrophils. Vascularization of the nodule was readily apparent.

The tumors produced by chemical carcinogen-treated cells were found to be undifferentiated mesenchymal tumors (1); those produced by cells treated with physical carcinogens were myxofibromas (3). When the incidence of tumor formation (Table 1) was correlated with growth in soft agar, it was readily apparent that the transformed cells able to grow in soft agar were subsequently able to produce tumors in mice. However, to date, chemical carcinogen transformed human foreskin fibroblasts that had not passaged through soft agar would not produce a tumor

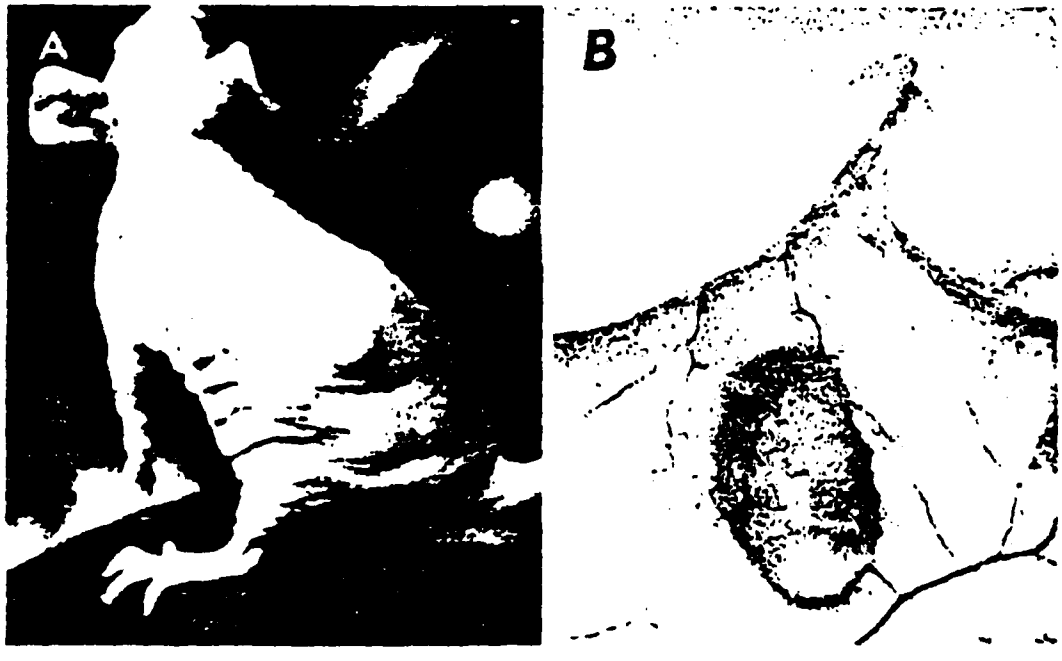

FIG. 5 *A, B.* Typical tumor in preirradiated nude mouse 6 wk after subcutaneous injection with 5×10^6 human fibroblasts transformed by Af-B_1.

when 5×10^6 or 2×10^7 cells were injected subcutaneously into the mouse. We did not observe a single spontaneous tumor in the mice preirradiated with ^{137}Cs, and karyological examination of tumors removed after 6 to 7 wk, confirmed that they were of human origin. Analysis of the chromosomes (2) showed that the human chromosomal constitution did not vary significantly from the diploid to near diploid number (46 to 49). Occasionally we observed a pseudodiploid arrangement (1).

Discussion

The data presented here were intended to illustrate the in vitro properties of human fibroblasts from neonatal foreskin that were transformed by chemical or physical carcinogens. We have found that human fibroblast populations must be treated with the carcinogen during or immediately prior to S if a carcinogen event is to be reproducibly induced. Cells can be arrested in G_1 by deleting from the growth medium the amino acids arginine and glutamine (1). Cell populations, upon entering a density arrested quiescent state (<5% labeled interphase nuclei), were serially passaged within 16 h into amino acid deficient medium. After 24 h (Fig. 1) the cells were fed with CM and 0.5 U/ml IN, and as the cells entered S, carcinogens were added. Carcinogens requiring specific transport, such as B(a)P, were added 12 h before the cells' entered S (11); compounds that are more carcinogenic at low pH, such as the bladder carcinogens *N*-hydroxyl-1-naphthylamine (*N*-OH-1-NA) and *N*-hydroxy-2-naphthylamine (*N*-OH-2-NA) (9,12), were added at pH 5 when the cells were in early S. Carcinogens exhibiting brief life spans were added in early S for up to 15 min: PrS, EMS, MAMA, 1,1 (unsymmetrical) dimethyl hydrazine (UDMH), methylmethane sulfonate (MMS). Other relatively stable carcinogens were kept in contact with the cells for 10 h as shown in Fig. 1. The physical carcinogens were applied during late S (5.0 j/m² at a fluence rate of 1.2 j/m²-sec for UV, and 56 rad/min for ^{137}Cs) (3). After the treated cell population had proceeded through S the cells were subpassaged immediately 1:2 into an 8X nonessential amino acid supplemented CM (8X medium). The cells were either seeded at high cell density to amplify the expression of the transformed phenotype or they were seeded at low cell density to evaluate the toxic effects of the carcinogens. We designated this phase of the carcinogenic process as the early stage. Other stages associated with the process occurred as the cells were passaged (Fig. 2).

In the past, randomly proliferating human cells in vitro rarely have been transformed following administration of a carcinogenic insult (13). Borek (14), using a synchronization method of serum deprivation, was able to transform KD cells by treating them in S following potentiation by either antipain or 17-β-estradiol. The use of 0.1% FBS to reduce cell proliferation prior to release and treatment with the carcinogens in S was avoided in our laboratory because under the reduced serum environment human diploid cells exhibit abnormal intracellular changes associated with the rough and smooth endoplasmic reticulum, expressed as changes in activating or lysosomal enzymes (15). McHale et al. (16) and Cristofalo (17), using diploid human lung cells, found that the transfer of logarithmically growing cells from 10% FBS-supplemented growth medium to 0.1% FBS-supplemented growth medium resulted in substantially fewer population doublings (premature senescence). The loss of the division potential could adversely affect the expression of the transformed phenotype at later stages in the carcinogenic process.

Normal human foreskin cells synchronized in G_1 by amino acid depletion have not exhibited shortened PDL (4). Synchronization of cells seems to be necessary for reproducible transformation by chemicals inasmuch as cell cultures treated in a confluent state are not transformed by the carcinogens. This apparent presensitization by amino acid deprivation was first reported by Grisham et al. (18). It seems also that this increase in sensitivity is further amplified by agents such as insulin, 17-β-estradiol, and phorbol myristate acetate.

We elected to control the environmental conditions after carcinogen treatment to select for the transformed cell population, rather than use extensive time in culture (14). The conditions included passaging the cells into CM supplemented with 8X nonessential amino acids and 2X vitamins, immediately following carcinogen treatment. After a period of 5 to 10 PDL the major portion of the cell population exhibited altered morphology. Continued passaging of the cells for 20 PDL yielded colonies that grew in 0.33% agar. By contrast, if the normal concentration of amino acids was used, we observed a 100-fold reduction in the proportion of cells that would grow in soft agar (unpublished data). The high amino acid

content in the growth medium amplified the transformed phenotype over a shorter time period and, although not absolutely necessary for expression of the transformed cells, it also reduced the proliferative capability of the normal cells. It is reasonable to conclude that supplementation of the CM with amino acids augments expression of the transformed phenotype. As has been observed in other transformation systems, cells that formed colonies with abnormal morphology would not immediately grow in soft agar or produce tumors.

We found that agar suitably prepared to support the growth of nasopharyngeal carcinoma cells (ATCC 443, American Tissue Culture Collection, Rockville, MD) or testicular carcinoma cells (gift from Dr. Ronald Trewyn, Ohio State University) would also support growth of the chemical carcinogen transformed cells (1). Optimum formation of colonies was in a LoCal medium –0.33% agar laid over a RPMI 1629–2% agar base. All of the transformed cells, except those treated with *N*-Ac-AAF, routinely produced colonies in soft agar. We observed only on one occasion that *N*-Ac-AAF treated cells would grow in soft agar; however, these colonies failed to produce tumors in mice. Colony formation in soft agar did not correlate with the degree of alteration in lectin agglutination profiles. However, all of the carcinogen-transformed cells that exhibited an altered profile of agglutination did produce colonies in soft agar. The largest colonies picked individually from the agar were propagated in CM with the 8X nonessential amino acid supplement and a suspension of 5×10^6 cells was injected into each mouse. A strong correlation existed between growth in soft agar and tumor formation, but the carcinogens that induced a high colony count in soft agar did not necessarily give rise to cell populations that would produce a high incidence of tumor formation in the mice (e.g., MAMA). This has also been documented in hamster embryo cells transformed by virus or chemicals or both (19). Conversely, 4-NQO treated cells had a low incidence of growth in soft agar but two of four mice developed tumors. All of the tumors that were removed from the animals and examined karyologically were characterized as human. The modal number of chromosomes was 46 to 49, and the chromosome distribution was diploid or pseudodiploid (1).

It is important to be aware that not all fibroblast cultures will be susceptible to carcinogen treatment. Occasionally, we have observed a totally refractory response to the carcinogen treatment, particularly with B(a)P. Refractory populations have been rare, however, when direct acting carcinogens such as MNNG or PrS were used.

Our procedure represents an inital attempt to define the progression of events that occur following exposure of human cells in vitro to different carcinogenic insults. Our data suggest that the cells pass through a similar progression toward neoplasm regardless of the class of carcinogen.

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Figure 1

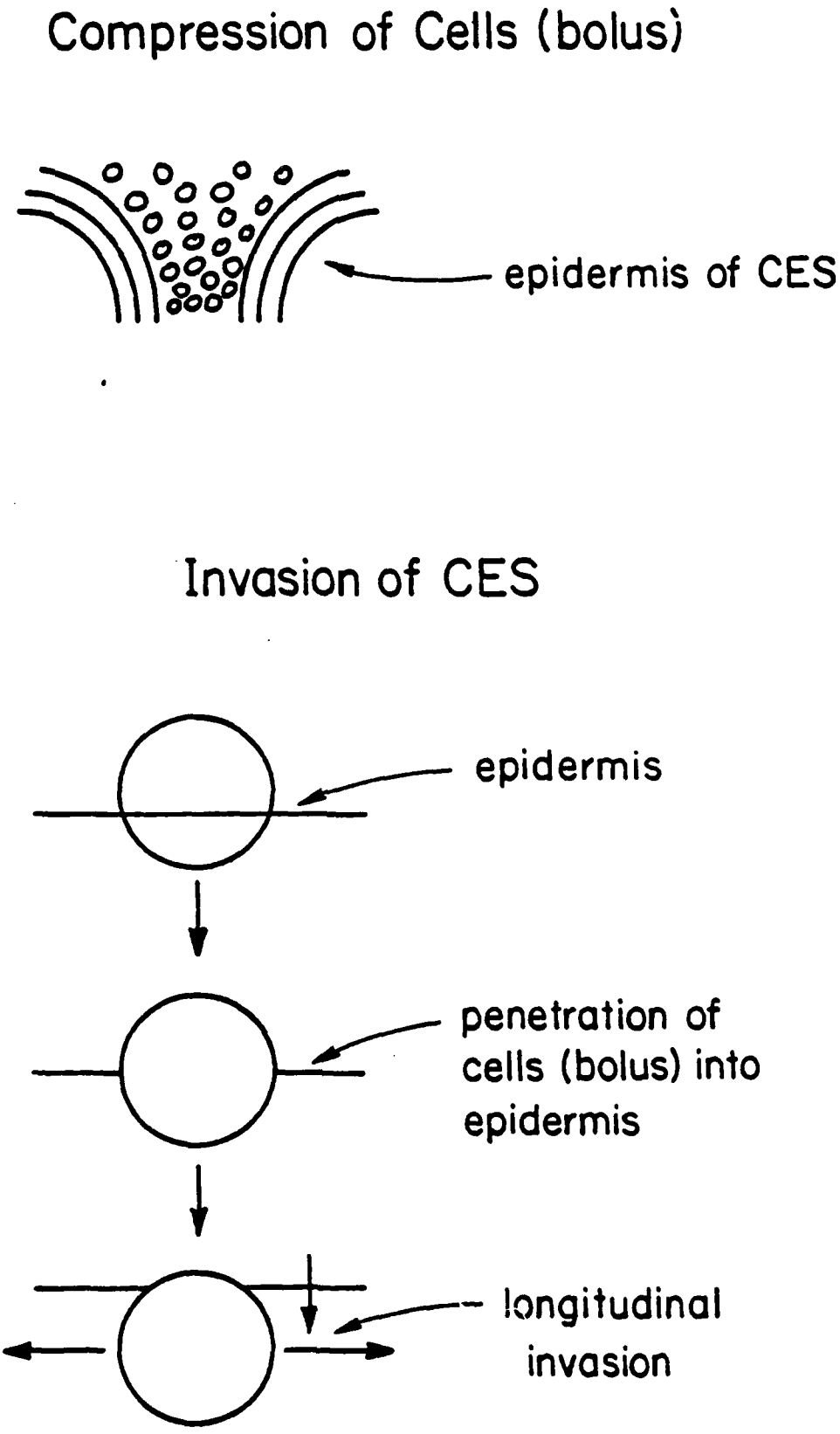

Compression of Cells (bolus)
epidermis of CES
Invasion of CES
epidermis
penetration of
cells (bolus) into
epidermis
longitudinal
invasion

Figure 1

Compression of Cells (bolus)

Invasion of CES

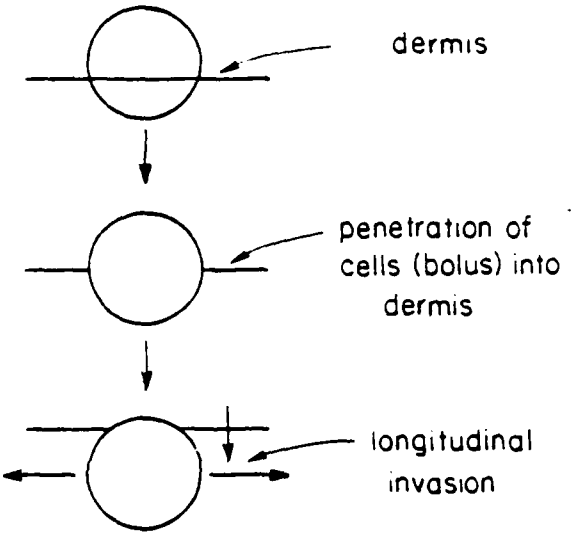
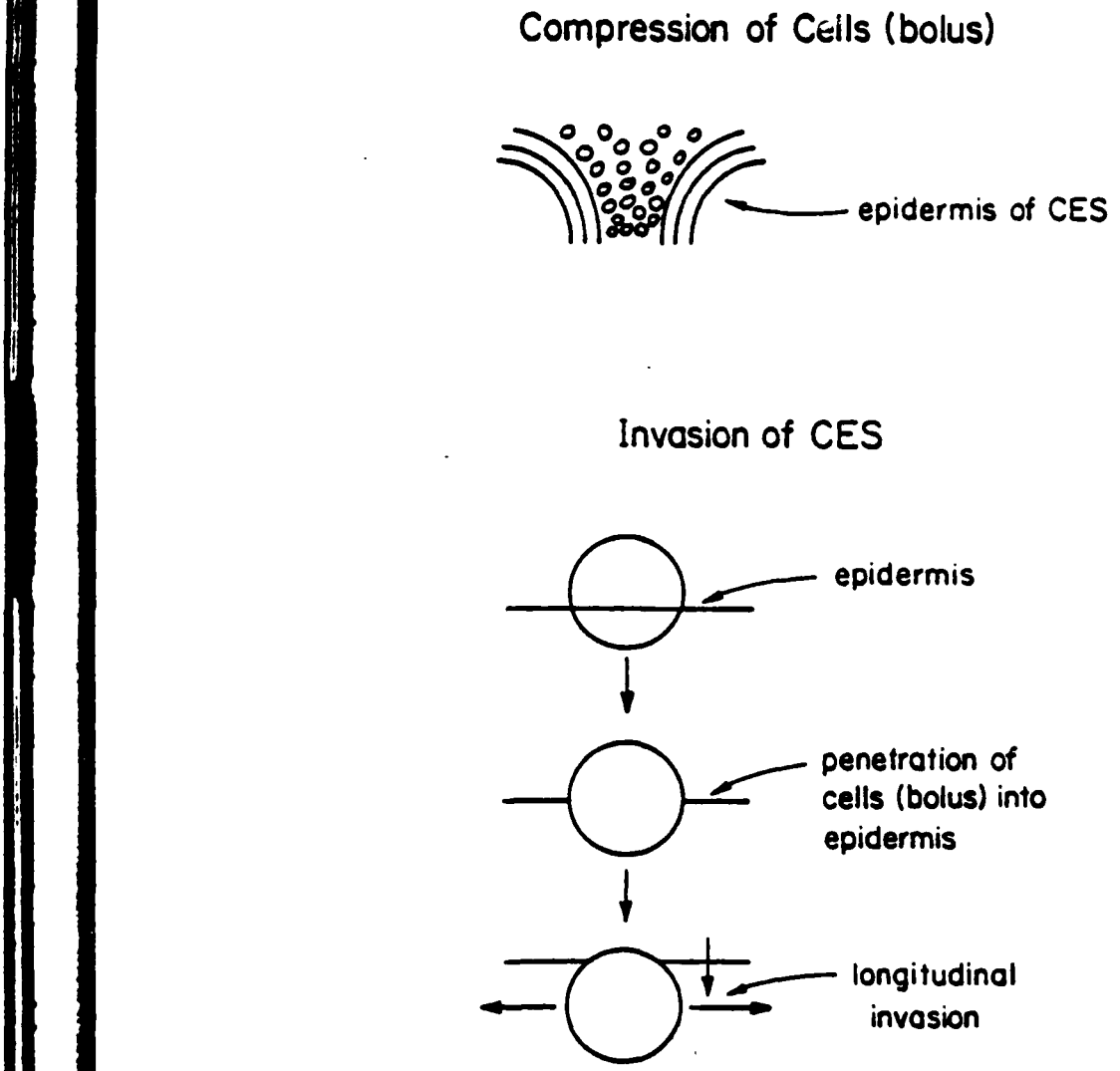


Figure 1


Compression of Cells (bolus)
epidermis of CES
Invasion of CES
epidermis
penetration of cells (bolus) into epidermis
longitudinal invasion

Reprinted from *Banbury Report 12: Nitrosamines and Human Cancer*

In Vitro Transformation of Cultured Human Diploid Fibroblasts

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The carcinogenicity of nitrosamines and nitrosamides was reported to be dependent upon the alkylation of macromolecules following the breakdown of the nitroso compounds (Loveless 1969). In mammalian rodent systems, a carcinogenic event occurred following methylation of the DNA (Magee and Hultin 1962; Magee and Farber 1962). Loveless (1969) suggested that the O^6 position of DNA-guanine is quantitatively alkylated by *N*-nitroso compounds. Gerchman and Ludlum (1973) suggested that methylated-O^6-guanine is read not as a guanine but adenine, therefore, a misspelling occurs at a critical site in the DNA with a subsequent carcinogenic response. Subsequently, Goth and Rajewsky (1974) indicated that the persistence of this error was the most important step in the carcinogenesis process and that the alkylation must persist until the DNA replicates semiconservatively and the daughter cells received this aberrant DNA.

In human cells treated with a carcinogen (Milo et al. 1978a, 1978b) we recognized that damage to DNA by these agents was repaired quickly by error-free repair systems (Milo and Hart 1976). Over the years attempts to reproducibly transform randomly proliferating human diploid cell populations has met with failure. However, we describe a program for the induction of carcinogenesis in vitro with human diploid fibroblasts using nitroso compounds as the instruments to deliver a carcinogenic insult. The biological endpoint measuring the carcinogenic insult will be an expression of anchorage-independent-growth, cellular invasiveness and neoplasia in a xenogeneic host.

METHODS

Chemicals

The chemicals of interest for this study are dimethylnitrosamine, (DMN, $CH_3N(NO)CH_3$); diethylnitrosamine (DEN, $CH_3CH_2N(NO)CH_3$); *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG); ethylnitrosourea (ENU, $C_2H_5N(NO)CONH_2$); and methylnitrosourea (MNU, $CH_3N(NO)CONH_2$). These chemicals

were furnished by the NCI Chemical Repository DCCP-NCI for this study sponsored by the National Cancer Institute. The modulators were obtained from commercial sources: insulin (Sigma Chemical, St. Louis, MO), phorbol myristate acetate, (Consolidated Midland Corp., Brewster, NY) and anthralin, (Sigma Chemical Co., St. Louis, MO).

Cell Cultures

Neonatal foreskin fibroblast cell populations were prepared as described previously, (Riegner et al. 1976; Oldham et al. 1980). Briefly, the fibroblasts were separated immediately from the mixed culture by selective detachment from the epithelial cells attached to a plastic substratum. The fibroblast cultures were passaged routinely using 0.1% trypsin and the subsequent cultures were maintained on Eagles-minimum essential medium (MEM) prepared with Hanks' balanced salt solution, 25.0mM HEPES buffer (GIBCO, Grand Island, NY) at pH 7.2. Additional supplements were added as needed, (Riegner et al. 1976). The cultures were incubated in an atmosphere of 4% CO_2-enriched air atmosphere at 37°C.

Transformation Procedure:

Toxicity Protocol

The transformation procedure is broken down into two distinct operational procedures: the cytotoxicity evaluation of each suspect carcinogen and the transformation protocol. Each chemical regardless of its structural similarity must be evaluated for its cytotoxic effects. Foreskin fibroblasts were isolated from the tissue (Allred et al. 1982; Oldham et al. 1980) and seeded at the first passage at 40 cells/cm^2 in MEM supplemented with growth additives, (Milo and DiPaolo 1978) Complete Media V (CMV), and incubated at 37° in a 4% carbon dioxide-enriched air environment and 10% FBS. The chemicals of interest are added to the populations for a 16-hour period at different concentrations of the chemical. The experimental medium was removed and replaced with MEM supplemented with 20% FBS, (Milo and DiPaolo 1980; Oldham et al. 1980). Two weeks later the cultures were fixed in 10% phosphate-buffered formalin and were stained with hematoxylin and eosin, and enumerated manually or on a differential image optical analyzer (Gavino et al. 1982). The data is expressed as relative colony forming efficiency i.e. the number of colonies 10 cells in size or larger that formed within 21 days of concluding treatment divided by the number of colonies that form in the untreated populations X 100.

Transformation Protocol

The concentrations of the compound of interest found to give an effective cytotoxic dose of 50%, 25% or noncytotoxic dose on cells at 40 cells/cm^2 were used

as the concentrations of choice to initiate the transformation process. We run every treatment in triplicate (i.e., on 3 different cultures derived from an initial culture prior to population doubling PDL 6). The randomly-logarithmically growing cultures at 5000 cells/cm^2 are seeded into a Dulbecco's Modified MEM minus arginine and glutamine and supplemented with dialyzed-FBS for 16 hours. The 2-hour-initiated thymidine radiolabeling index decreases from 18-23% to 0.1% to 0% in that time, (Milo and DiPaolo 1980; Oldham et al. 1980). At that time the cells are released from the G_1 block by the readdition of CMV-10% FBS containing 2mM glutamine and 1mM arginine plus either modulator 10U/ml insulin, 0.1g/ml anthralin or 5×10^{-7}M PMA. Ten hours following release from the G_1 block the cells exhibit S phase entry. The carcinogens of interest were added and left on for 12 hours. At the conclusion of the treatment the experimental medium was removed and replaced with CMV supplemented with 10% FBS. The cells were allowed to recover for 2 days and then were split 1:2 into CMV containing 2x vitamins, 8x nonessential amino acids and 20% FBS, hereafter referred to as 8x medium. When these treated cultures reached 80% to 90% confluent density they were serially passaged 1:10 into 8x medium until PDL-20.

CHARACTERIZATION OF TRANSFORMED CELLS:

Anchorage Independent Growth

The carcinogen populations at PDL 20 were seeded at 50,000 cells in 2ml of 0.33% agar, (in a 25cm^2 well) prepared in Dulbecco's LoCal medium (Biolabs, Northbrook, Ill) supplemented with additives, (Milo et al. 1981c) and 20% FBS. This cell suspension was layered over 5ml of a 2% agar base prepared in RPMI 1629 medium (GIBCO) plus additives, (Milo et al. 1981a,c). The seeded cultures were not disturbed for 1 week and were subsequently observed on a weekly basis for 3-4 weeks. Cultures were scored as positive when colonies of > 50 cells were observed. Colonies were removed and reestablished in culture. After attachment and growth to ~20% density the cells were trypsinized and reseeded to distribute evenly over the substratum.

Cellular Invasiveness

The carcinogen-treated population that formed colonies in soft agar were reestablished in culture and subsequently evaluated for tumor potential, (Noguchi et al. 1978; Milo et al. 1981c), and chick embryonic skin (CES) in vitro. The CES organ culture was modified to optimize sensitivity to the transformed cells and frequency of success for a rapid assay for cellular neoplasia. Eggs were incubated for 9-10 days in a humidified egg incubator. The embryos were removed from the eggs, the skins separated from the dorsal part of the embryo and placed on an agar base containing 10 parts of 1% agar in Earle's balanced salt solution, 4 parts FBS, and 4 parts chick embryo extract. The treated cells

250,000 in number contained in 0.04μl of MEM were added to the CES organ culture. These cultures were incubated in a humidified incubator at 37°C in a 4% CO_2 enriched air atmosphere. On day 4 the skins were removed and fixed in Bouin's solution. The stained 5μM sections on slides were examined by light microscopy (Milo et al. 1981a).

Tumor Growth in Nude Mice

Treated populations that exhibited anchorage independent growth were evaluated in 6 week old nude mice (Sprague-Dawley). The mice were irriadiated with 137CS source at 450 RAD whole body irradiation 48 hours prior to subcutaneous injection of 5×10^6 cells. Six weeks later the tumors were counted and the incidence of tumor formation recorded, (Milo et al. 1981a).

RESULTS

The cell population treated with the individual compounds included horcin were treated at equivalent cytotoxic doses to transform the human foreskin fibroblasts. It has been found that treatment regimens for transformation protocols were more reliable when the populations at risk to the suspect carcinogenic agent were treated at $ED_{10,25 \text{ or } 50}$ toxic doses or noncytotoxic equivalent doses rather than chemical equivalent doses. In all experiments where toxic values exceeded the ED_{50} values the incidence of transformation as measured by anchorage independent growth and cellular invasiveness tended to drop to zero, (control values). Once the toxicity of these compounds has been evaluated then the toxicity of the modulators has to be determined. All modulators were used at ED_0 doses i.e. a noncytotoxic dose. The problem one was faced with in these comparisons were the integrated affects that the modulators have on cell permeability. This was why we used these modulators on the cells at exceedingly low concentrations to elicit the proper response without interfering with the cytotoxic dose of the carcinogens of interest (Table 1).

Table 1
Cytotoxicity of Nitroso-amine Derivatives Determined on Human Foreskin Fibroblasts In Vitro

Chemical Compound	Cytotoxic Effect ED 50 in 4 (μg/ml)
DMN	0.001
DEN	0.01
MNU	29.0
ENU	44.0
MNNG	0.1

These concentrations represent the cytotoxic dose that yielded a 50% inhibition of the relative cloning efficiency. Data from Allred et al. 1982).

Early Stage

Treated populations responded to the carcinogenic insult in a different predictable manner than populations just responding to toxic insults. First, for example, populations responding to toxic levels of modulators while exhibiting selective changes in plasma membrane permeability, did not exhibit altered lectin agglutination profiles as seen by populations responding to a carcinogenic insult.

Once carcinogen-treated populations exhibited these features they were serially passaged for 20 population doublings (PDL) before seeding in soft agar.

Transitional Stage

Using the growth medium we described under transformation protocol (Material and Methods section), the optimum time for seeding the treated populations in soft agar was 20 PDL following treatment with the carcinogen. Second, it was interesting to note that at this PDL, DMN-, DEN-, and MNNG-treated populations exhibited anchorage independent growth while ENU- and MNU-treated populations did not exhibit this feature. DMN induced colony formation in soft agar of 13 colonies/10^5 cells; DEN, 8 colonies/10^5 cells and MNNG, 1 colony/10^5 cells. The colonies 50 cells in size or larger were removed and reseeded in flasks. At a 80% preconfluent density the cells were seeded onto CES and 4 days later evaluated (Table 2). The treated populations that exhibited anchorage independent growth also exhibited cellular invasiveness.

Table 2
Evaluation of Different Nitrosamine-Treated Cell Populations for Anchorage-Independent Growth, Cellular Invasiveness, and Tumor Incidence

Chemical compound	Cytotoxic effect (ED 50 μg/ml)[a]	Cellular invasiveness[b]	Anchorage-independent growth[c]	Tumor incidence[d]
DMN	0.001	+	13	2/6
DEN	0.01	+	8	3/8
MNU	29.0	-	0	N.D.
ENU	44.0	-	0	N.D.
MNNG	0.1	+	1	3/5

[a]Represents the cytotoxic dose that yielded a 50% inhibition of the relative cloning efficiency.

[b]A positive response indicates that out of 6 CES organ cultures one or more sections upon examination by the pathologist exhibited cellular invasiveness.

[c]Colonies 50 cells or more in size were scored as positive when counted 21 days post-seeding in the soft agar overlayer. Each 25 cm^2 well was seeded with 50,000 cells. (Methods section).

[d]The numerator represents the number of positive takes (tumors) over the number of mice injected with 5×10^6 treated cells. (Methods section).

AD-A121 284 CHEMICAL CARCINOGEN (HYDRAZINE ET AL) INDUCED 2/2
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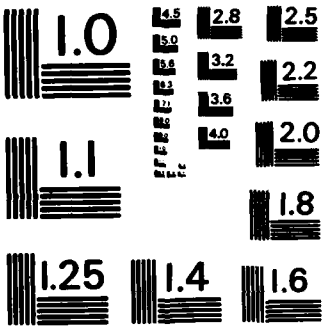

Late Stage

At this time the different treated populations were injected in nude mice and the incidence of tumor formation noted. Injected DMN-treated cells elicited an incidence of tumor formation in 2 out of 6 mice; DEN treated cells 3 out of 5. The tumors were examined by histopathology and interpreted to be undifferentiated mesenchyal tumors. The cellular tumors evaluated from the CES were described as simulated firbosarcomas. This definition was used because the pathologist was describing cellular invasion into CES, an organ culture, in vitro. The two interpretations are not incompatible.

DISCUSSION

The data here were presented to illustrate the sequencing that can optimize the program of human foreskin fibroblast response to a carcinogenic insult in vitro. These compounds were evaluated for their carcinogenic potential appear to require cell proliferation for the fixation of the carcinogen damage. Once the insult is fixed, a program of selective expression of the initiated cells occurs over a protracted time period, 20 PDL, followed by anchorage independent growth of the transformed cells.

A similar selection pressure is also expressed by the cell population prior to carcinogen treatment, i.e. cells passaged in culture > 10 PDL are refractory to a carcinogenic insult (Sutherland, et al. 1980; Milo et al. 1981c; Zimmerman and Little et al. 1981). These observations are not unusual in themselves, for ex example, we have seen the same refractoriness exhibited by human foreskin populations to feline sarcoma virus-directed transformations in vitro (Milo et al. 1981b) at high PDL. These vector-directed transformations also exhibit multistage (early, transitional, and late stage of expression of carcinogenesis) carcinogenesis exhibited by the nitrosamine-transformed cell populations. An interesting feature of the chemical carcinogen-induced transformation of human cells as seen by Silinskas et al. (1981) was that they used a different medium to culture their cells in soft agar and observe colony formation around 8-13 PDL following carcinogen treatment rather than 20 PDL. Zimmerman and Little (1981), and Sutherland et al. (1980) observed also that cells treated at PDL > 10 were refractory to the carcinogenic insult. Recently, Tejwani et al. (1982) demonstrated that DNA-adducts formed by benzo[*a*]pyrene [B[*a*]) or B[*a*]P 9,10-ene diol epoxide anti form of B[*a*]P in susceptible populations were qualitatively similar to the principle adduct formed in the refractory cells i.e. B[*a*]P 9,10 ene diol epoxide-deoxyguanosine adduct.

It is our contention that optimum sensitivity occurs in S-phase to the carcinogen insult and that the response can be amplified by the addition of modulators (Milo and DiPaolo 1980). These events are followed by the persistence of the adducts during the critical point of the fixation of the carcinogenic insult immediately prior to and during the early phase of replication of DNA in S-phase. These events are followed by a selection process for the early

stage of carcinogenesis, i.e., altered lectin agglutination profiles (Milo et al. 1981c) and expression of the early transformed phenocopy.

As pointed out by Zimmerman and Little (1981) and reported by Kapp and Painter (1979) the rate of scheduled DNA synthesis in human cells is proportional to the number of functional replicons in S at a given time. They also point out that repair of DNA damage is not extensive over the treatment period.

Following from this we hold the cells in G_1 block prior to S-phase entry in the presence of the exogenously supplied modulators which may amplify the number of replication sites and expose carcinogenic site(s) that would otherwise be masked. It may not require a permanent change in a molecular event such as that described or such as deficient DNA repair synthesis or persistence of damage, but it may require a change in events of the replicon similar to that seen in normal differentiation patterns. As we presently know, human nasopharyngeal carcinoma tumors are exceedingly difficult to grow in vitro. They purportedly differentiate in culture and produce keratin and enter an amitotic stage, (pers. comm. R. Glaser). Milo et al. (1981a) showed that carcinogen-initiated keratinocytes when seeded directly into soft agar produce colonies. They express anchorage-independent growth. However, when carcinogen-initiated keratinocytes are transferred to culture conditions following carcinogen treatment they tend to differentiate and proceed into an amitotic stage. It is possible that there is a program for carcinogenesis in human cells that when followed rigorously can lead to a reproducible expression of a carcinogenic event. This program is comprised of a sequence of events that must occur prior to induction, i.e. activation, selective adduct formation, error-prone repair, protein modulation. These events are then followed by a program of events leading to a transformed phenocopy exhibiting anchorage-independent growth, cellular invasiveness and neoplasia.

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COMMENTS

CONNEY: I have heard of human cells that were transformed in vitro by chemicals, that by all criteria in vitro are transformed cells, but when they are put into nude mice, they start to grow and form a nodule, and then the nodule regresses. There is really something different about this cell. It may be a quasimalignant cell. Have you seen these results?

MILO: No! Not after a 4-week interval has elapsed—before tumors are counted.

CONNEY: Is there something in the environment of a so-called quasimalignant cell that can cause that cell to revert back to normal?

MILO: What is the normal phenotype that a cell should regress back towards to be described as a "normal" cell? Tumor material taken from soft-tissue tumors of humans behaves in a nude mouse system in a similar *manner as that described for in vitro transformed cells.*

PREUSSMANN: I was astonished at the vast differences in the cytotoxicity of your compounds. The nitrosoureas were less cytotoxic and the nitrosamines were very cytotoxic. You would expect the reverse order, because nitrosoureas are used in cancer chemotherapy as cytotoxic agents. Could it be that the solutions that you used in your experiments deteriorated after you used it? Those are unstable compounds. Have you any explanation for that?

HICKS: We would confirm the same observation in bladder cells. We cannot kill human bladder cells in culture with MNU.

MICHEJDA: MNNG in human fibroblasts is extremely cytotoxic. MNNG was also rather noncytotoxic in this experiment.

TANNENBAUM: Your effective concentration of DMN is 1 ng/ml. per milliliter. Many people have done experiments with DMN in a variety of cells. Usually they are up in millimolar concentrations before they begin to get effect. Has anyone characterized anything about the enzymology of these?

MILO: We are working on it right now.

PREUSSMANN: Could your solution of nitrosourea have been degraded after you it into your cells?

MILO: Chromatically speaking we know that what we put on in ~~there is what is to be using radiolabel~~. DMN or DEN ~~the compound~~ appears to be stable when evaluated by HPLC.

does not degrade

also

GOLDFARB: Is there any evidence really that we are seeing real invasion or infiltration by these tumor cells into the skin?

MILO: If we see this occurring you occasionally will see necrosis. The suppression phenomenon can be seen by virtue of the fact that what will happen is that the epidermal type of cells, when they are put on the CES, will have this kind of feature. You will see compression all through the CES when the cells are layered on the skin. When you see invasion, what is seen when subsequently thick slices are taken, is shown in the accompanying figure:

When the slides are positive you will see the cells growing down into the CES, and longitudinally under the dermal layer of CES. So we take sequential slices in this area to try to address the problem that you are talking about.

END

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